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(54) Title: CELLULAR REPROGRAMMING IN PEPTIDE HYDROGEL AND USES THEREOF

(57) Abstract: The present invention provides compositions featuring cells, e.g., progenitor cells, stem cells, and their progeny encapsulated within self-assembling threedimensional peptide hydrogel structures (scaffolds). The scaffolds provide a nanoscale environment. Thus the invention provides a nanoscale environment scaffold encapsulating cells. According to certain embodiments of the invention the progenitor cells and/or their progeny are able to differentiate or transdifferentiate within the structures. According to certain embodiments of the invention the cells are or differentiate into liver lineage cells, neural lineage cells, or pancreatic lineage cells. The peptide hydrogel environment renders the cells permissive for instruction by differentiation-enhancing agents such as growth factors or extracellular matrix components. The invention provides an in vitro culture system for growing stem and progenitor cells, for inducing their differentiation and transdifferentiation, and for studying their properties; an in vitro culture system for growing hepatocyte-like cells and/or mature hepatocytes, which may be used, for example, to propagate hepatotropic viruses; a system for generating insulin secreting cells; a system for controlling and manipulating cell differentiation and transdifferentiation in vitro, from which cells can be extracted and then either maintained in vitro or administered to a subject; a system for controlling and manipulating cell differentiation and transdifferentiation in vitro, and assay systems for testing compounds. The invention provides methods of treating disease by implanting peptide hydrogel structures containing cells or cells extracted following culture in peptide hydrogels into the body.

CELLULAR REPROGRAMMING IN PEPTIDE HYDROGEL AND USES THEREOF

GOVERNMENT SUPPORT

This invention was supported by grants awarded by the National Institutes of Health, and the government of the United States has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S.S.N. 09/778,200, entitled "Peptide Scaffold Encapsulation of Tissue Cells and Uses Thereof", filed Feb. 6, 2001, and provisional application U.S.S.N. 60/305,379 entitled, "Liver Cellular Reprogramming in Peptide Hydrogel and Uses Thereof", filed July 13, 2001, both of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to techniques for inducing the differentiation and transdifferentiation of progenitor cells. The invention includes compositions and methods involving encapsulation of various cells including progenitor cells or stem cells. In particular, the invention involves the use of nanoscale materials such as peptide hydrogels in such techniques.

BACKGROUND OF THE INVENTION

During embryonic development all animal tissues and organs originate from a population of undifferentiated and actively dividing cells. Ultimately these cells give rise to the wide variety of differentiated cell types found in an adult organism. As an embryo develops, cells that have acquired a particular fate proliferate, allowing the growth of tissues and organs. However, even after an animal is fully grown many tissues and organs are able to maintain themselves despite cell loss through natural cell attrition or injury. The remarkable capacity of an embryo to develop and diversify and of adult organs and tissues to regenerate arise due to the presence of stem cells. Stem cells possess the ability to renew themselves, i.e., to

divide and generate additional self-renewing stem cells, and also to give rise to cells that are able to differentiate along various differentiation pathways.

There has been considerable interest in identifying, isolating, and propagating stem cells, and major breakthroughs have occurred. Stem cell technology has led to the development of important research tools such as transgenic mice and also enabled medical treatments such as bone marrow transplants and gene therapy for certain diseases. From a research point of view, an enhanced understanding of stem cell biology is likely to have a significant impact on areas ranging from development to aging and cancer. Stem cell technology also promises to yield numerous additional medical applications. For example, stem cells could prove extremely useful in tissue engineering, a multidisciplinary field that seeks to develop *in vitro* cell and cell/material systems and structures that can be used to repair or replace damaged or diseased tissues or organs.

While significant advances have been made, major challenges remain before the potential utility of stem cells and other progenitor cell types that may lack self-renewal capacity and/or have a more restricted differentiation potential can be fully realized. Understanding of the factors that trigger and control stem cell division and differentiation remains incomplete. It is thought that such cells respond to a variety of cues, which may include growth factors, cell-cell contacts, and structural features of the extracellular environment among others. Many studies of stem cells involve the removal of cells from one animal followed by transplantation into another individual and subsequent examination of the ability of the transplanted cells to give rise to a diversity of cell types. While useful in terms demonstrating the existence and potential of stem cells, such approaches are limited.

In order to fully exploit the unique features of stem cells and other progenitor cell types, it is desirable to be able propagate such cells *in vitro*. In addition, it is desirable to be able to control and manipulate the processes of self-renewal and differentiation so that a variety of cell types can be generated. Therefore, there exists a need for improved cell culture systems, techniques, and compositions for maintaining stem cells and progenitor cells and for altering and controlling their division and differentiation. In addition, there exists a need for culture systems and compositions for cell culture that would allow the harvesting of cultured cells after a

period of cell growth and/or differentiation in the culture system. Furthermore, there exists a need for culture systems and compositions for cell culture that could be implanted into the body, e.g., for tissue engineering purposes. In particular, there exists a need for three-dimensional culture systems that might mimic the natural cellular environment more closely than the two-dimensional surface of traditional culture systems.

Many previous efforts to develop such systems have involved the use of materials such as proteins and peptides obtained from animal sources. Such materials have a number of disadvantages as compared with synthetic materials. For example, they present an increased risk for the transmission of disease. In addition, it can be difficult to ensure that different preparations of material have a consistent, reproducible composition. Even when it is possible to achieve consistency with respect to the known components of a material isolated from a natural source, it is hard or impossible to ensure that unknown, perhaps as-yet unidentified components that may affect cell properties, are excluded. Thus there remains a need for synthetic compositions and materials for cell culture and tissue engineering purposes. In particular, for applications involving implantation into the body, there remains a need for such compositions and materials that elicit no or minimal immune or inflammatory response and for compositions and materials that are degradable within the body.

The regenerative capacity of the body is particularly striking in the case of certain organs such as the liver, which is well-known to possess extensive capacity to regenerate after insults ranging from partial hepatectomy to toxin-induced injury. However, it has not been possible to satisfactorily maintain differentiated hepatocytes in culture. In addition, understanding of liver stem cells remains limited, and the potential of liver-derived cells has not been fully explored. Therefore, there is a need for the development of improved cell culture systems, techniques, and compositions for the propagation of liver progenitor cells and stem cells and for exploiting their differentiation and transdifferentiation potential. In addition, there is a need for the development of improved cell culture systems, techniques, and compositions for the propagation of various other progenitor or stem

cells, including neural lineage cells, and for exploiting their differentiation and transdifferentiation potential.

SUMMARY OF THE INVENTION

The invention represents the convergence of research in the fields of progenitor and stem cells and biomaterials. The inventors have discovered that self-assembling peptide hydrogel structures derived herein support the differentiation and transdifferentiation of cells. The invention provides a macroscopic structure comprising amphiphilic peptides, wherein the peptides comprise substantially equal proportions of hydrophobic and hydrophilic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure, and a population of progenitor cells. In certain embodiments of the invention the progenitor cells are encapsulated in the structure. In certain embodiments of the invention the macroscopic structure further comprises a differentiation-enhancing or transdifferentiation enhancing agent, e.g., a growth factor or extracellular matrix (ECM) component. In general, as used herein, the term "differentiation enhancing agent" shall be understood to include agents that enhance, stimulate, or contribute to differentiation and/or transdifferentiation. According to certain embodiments of the invention the peptide structure renders the cells permissive for instruction by the agent. In certain embodiments of the invention the progenitor cells are derived from the liver.

The invention further provides methods of culturing cells comprising (i) providing progenitor cells or stem cells, and (ii) contacting the progenitor cells with a cell culture material comprising amphiphilic peptides, wherein the peptides comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure. The progenitor cells or stem cells may be encapsulated within the peptide structure by (i) incubating the peptides and the progenitor cells in an aqueous solution comprising an iso-osmotic solute; and (ii) adding an electrolyte to the solution sufficient to initiate self-assembly of the peptides into a beta-sheet macroscopic structure, whereby the cells are encapsulated by the formation of the scaffold. The method may further include adding a

differentiation-enhancing or transdifferentiation enhancing agent, such as a growth factor or ECM component, either before self-assembly or to media in which the cell/peptide structure is incubated. According to certain embodiments of the invention the agent causes a portion of the progenitor cells or their progeny to transdifferentiate. According to certain embodiments of the invention the macroscopic structure renders at least a portion of the progenitor cells permissive for instruction by the agent. In certain embodiments of the invention the progenitor cells are derived from the liver.

Generally, the invention provides methods of culturing cells comprising encapsulating the cells in a three-dimensional nanoscale environment scaffold. According to certain embodiments of the invention the nanoscale environment scaffold comprises a protein or peptide hydrogel. The hydrogel may be a self-assembling peptide hydrogel as described herein. According to certain embodiments of the invention the peptides comprise amphiphilic peptides, wherein the peptides comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure. According to certain embodiments of the invention the nanoscale environment scaffold comprises nanofibers. The nanofibers may be comprised of self-assembling peptides, e.g., any of the peptides described herein. According to certain embodiments of the invention the cells comprise or consist of progenitor cells. According to certain embodiments of the invention the cells comprise or consist of stem cells. According to certain embodiments of the invention the cells comprise liver-derived cells or cells derived from neural tissue. According to certain embodiments of the invention the cells comprise progenitor cells or stem cells that have been instructed or induced to differentiate. For example, the cells may be instructed or induced to differentiate along a liver cell lineage pathway, along a neural lineage pathway, or along a pancreatic lineage pathway. The cells may comprise liver cells (e.g., liver progenitor cells, liver stem cells, hepatocytes, oval cells, bile duct cells), neural lineage cells (e.g., neurons or glia), and/or pancreatic lineage cells (e.g., endocrine cells such as β, α, γ, or δ cells or exocrine cells).

The invention further provides nanoscale environment structures and scaffolds encapsulating cells. These terms are generally used interchangeably herein. The scaffolds encapsulating cells may be prepared according to the methods described herein or variations thereof. According to certain embodiments of the invention the nanoscale environment scaffold comprises a protein or peptide hydrogel. The hydrogel may be a self-assembling peptide hydrogel as described herein. According to certain embodiments of the invention the peptides comprise amphiphilic peptides, wherein the peptides comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure. In certain embodiments of the invention the nanoscale environment comprises or consists of an artificial material. According to certain preferred embodiments of the invention, the artificial material comprises or consists of a material not naturally found in the body. Artificial material also encompasses certain materials obtained by isolating and processing substances produced by a living source. However, a material that remains substantially intact and substantially retains the structure in which it is naturally found within the body of an organism is not considered an artificial material. Any of a variety of artificial materials may be used.

According to certain embodiments of the invention the nanoscale environment scaffold comprises nanofibers. The nanofibers may be comprised of self-assembling peptides, e.g., any of the peptides described herein. According to certain embodiments of the invention the cells comprise isolated cells, e.g., cells that are not in their natural environment within the body of a subject. For example, the cells may comprise cells that have been removed from a subject. Such cells may have been cultured following removal prior to encapsulation. The cells may comprise a cell line. According to certain embodiments of the invention the cells comprise or consist of progenitor cells. According to certain embodiments of the invention the cells comprise or consist of stem cells. According to certain embodiments of the invention the cells comprise liver-derived cells or cells derived from neural tissue. According to certain embodiments of the invention the cells comprise stem cells or progenitor cells that have been instructed or induced to

differentiate. The cells may be instructed or induced to differentiate along a liver cell lineage pathway, along a neural lineage pathway, or along a pancreatic lineage pathway. The cells may comprise liver cells (e.g., liver stem cells, liver progenitor cells, hepatocytes, oval cell, bile duct cells), neural lineage cells (e.g., neurons or glia), and/or pancreatic lineage cells (e.g., endocrine cells such as β, α, γ, or δ cells or exocrine cells). Of course the nanoscale environment scaffold encapsulating cells may encapsulate a combination of different cell types.

The invention provides methods of treating an individual comprising (i) identifying an individual in need of treatment; and (ii) administering a nanoscale environment scaffold encapsulating cells to the individual. The nanoscale environment scaffold encapsulating cells may be any of the nanoscale environment scaffolds described above. In particular, the nanoscale environment scaffold encapsulating cells may comprise or consist of stem cells or progenitor cells. According to certain embodiments of the invention the cells comprise liver cell lineage cells, neural lineage cells, or pancreatic lineage cells. According to certain embodiments of the invention the cells comprise stem cells or progenitor cells that have been instructed or induced to differentiate. The cells may be instructed or induced to differentiate along a liver cell lineage pathway, along a neural lineage pathway, and/or along a pancreatic lineage pathway. The cells may comprise liver cells (e.g., liver stem cells, liver progenitor cells, hepatocytes, oval cells, bile duct cells), neural lineage cells (e.g., neurons or glia), and/or pancreatic lineage cells (e.g., endocrine cells such as β, α, γ, or δ cells or exocrine cells).

As is well known in the art, many cell types are found within a three-dimensional environment within the body. Such a three-dimensional environment may include extracellular matrix components, e.g., collagen, fibronectin, laminin, etc. The physical and chemical properties of such three-dimensional environments may vary, which may affect the ability of the environment to support the growth of different cell types. According to certain embodiments of the various inventions provided herein, the cells comprise cell types that are normally found within a three-dimensional environment within the body. According to certain embodiments of the inventions, the cell types preferably are not naturally found in a cartilaginous

environment within the body. According to certain embodiments of the invention the cells preferably do not include chondrocytes or chondrocyte precursor cells.

In addition, the invention provides methods of treating an individual comprising (i) identifying an individual in need of treatment; and (ii) administering cells to the individual, wherein the cells have been induced to differentiate or transdifferentiate by culturing them encapsulated in a cell culture material comprising amphiphilic peptides, wherein the peptides comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure. According to certain embodiments of the invention the cells are exposed to a differentiation or transdifferentiation enhancing agent. The cells may be extracted from the structure prior to administration or the cell/peptide structure may be introduced into the individual.

In another aspect, the invention provides a cell culture kit comprising (i) amphiphilic peptides, wherein the peptides comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure; and (ii) instructions for initiating self-assembly of the peptides into a macroscopic structure. The kit may further comprise at least one element selected from the group consisting of: a population of cells, cell culture medium, a predetermined amount of a growth factor, a predetermined amount of an electrolyte, instructions for encapsulating cells within a peptide hydrogel structure and for other uses of the system, instructions for inducing cells to differentiate or transdifferentiate within the scaffold, a vessel in which the encapsulation may be performed, a liquid in which the peptide can be dissolved, an electrolyte for initiating peptide self-assembly, medium for tissue culture, and one or more differentiation-enhancing agents.

In another aspect, the invention provides an assay system comprising a population of cells derived from the liver or their progeny, wherein the cells express a cytochrome P450 enzyme, and wherein the cells are encapsulated in a beta-sheet macroscopic scaffold comprising peptides that comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and

structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure. The assay system may further comprise a substrate for the cytochrome P450 enzyme.

In another aspect, the invention provides methods of testing a compound comprising steps of (i) contacting a population of cells derived from the liver or their progeny, wherein the cells express a cytochrome P450 enzyme, and wherein the cells are encapsulated in a beta-sheet macroscopic scaffold comprising peptides that comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure, with the compound; (ii) measuring activity of the cytochrome P450 enzyme; and (iii) comparing the level of activity of the enzyme in the presence of the compound with level of activity in the absence of the compound.

The invention further provides a method of testing a compound comprising steps of (i) contacting a population of cells derived from the liver or their progeny, wherein the cells express a cytochrome P450 enzyme, and wherein the cells are encapsulated in a beta-sheet macroscopic scaffold comprising peptides that comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure, with the compound, (ii) measuring activity of the cytochrome P450 enzyme; and (iii) comparing the level of activity of the enzyme in the presence of the compound with level of activity in the absence of the compound.

The present invention refers to various patents, patent applications, books, and publications in the scientific literature. The contents of all such items are incorporated herein by reference in their entirety. In addition, except as otherwise indicated, the present invention may employ standard cell culture techniques and media and standard molecular biological and immunological protocols such as are found in reference works such as Freshney. R. I., *Culture of Animal Cells: A Manual of Basic Technique*, 4th ed., John Wiley & Sons, New York, 2000; Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, NewYork, 2000; Harlow, E., Lane, E., and Harlow, E., (eds.) *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1998.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration showing the hypothetical interactions between peptides in an assembled peptide structure.

Figure 2 is a picture of assembled peptide structures formed in a variety of predetermined shapes.
Figure 2a shows a tape-shaped scaffold structure.
Figure 2b shows a string-shaped scaffold structure.
Figure 2c shows a sheet-like scaffold structure.

Figure 3 shows encapsulation of adult LPCs in an assembled peptide structure.
Figure 3a shows encapsulated LPCs immediately after encapsulation.
Figure 3b shows encapsulated LPCs two days after encapsulation.
Figure 3c shows spheroid formation by adult LPCs encapsulated in an assembled peptide structure four to five days after encapsulation.
Figure 3d shows the same spheroid as in Figure 3c, after staining for incorporation of BrdU into DNA.

Figure 4 presents data on CYP1A1 activity of LPCs growing under various culture conditions.
Figure 4a shows CYP1A1 activity of LPCs growing as a monolayer on a standard plastic culture dish.
Figure 4b shows CYP1A1 activity of LPCs spheroids growing in an assembled peptide structure two weeks after encapsulation.
Figure 4c is a graph showing CYP1A1 activity of LPC spheroids growing in an assembled peptide structure during a time course of two weeks, starting 24 hours after encapsulation.
Figure 4d is a graph showing comparative CYP1A1 activity of LPCs maintained under various culture conditions: monolayer on plastic dish with low (1%) serum concentration (serum starvation); spheroid culture obtained by growing LPCs in

liquid culture at high density; spheroid culture in assembled peptide structure growing in DMEM with 10% FBS; spheroid culture in assembled peptide structure growing in HGM.

Figure 5 shows LPCs growing in an assembled peptide structure after staining for various neuronal markers.
Figure 5b shows LPCs stained for Nestin.
Figure 5d shows LPCs stained for β-tubulinIII.
Figure 5f shows LPCs stained for NeuN.
Figure 5h shows LPCs stained for GFAP.
Panels 5a, 5c, 5e, and 5g are corresponding phase contrast images.

Figure 6 shows Nestin (6b, 6f) and BrdU (6d, 6h) staining of neuronal-precursor-like cells arising from LPC clusters cultured in an assembled peptide structure in HGM in the presence of EGF (6a-6d) or EGF plus NGF (6e-6h). 6a, 6c, 6e, and 6g are phase contrast images of 6b, 6d, 6f, and 6h.

Figure 7 shows the phenotype of LPC cells cultured on laminin-coated plates in HGM containing EGF plus NGF one week after extraction from assembled peptide structures.
Figure 7a shows cells that assumed a classical hepatocyte shape.
Figure 7b shows the same cells as in 7a after staining for CYP1A1 activity.
Figure 7c shows cells that assumed a flat, expanded shape with some processes.
Figure 7d shows the same cells as in 7c after staining for GFAP.
Figure 7e shows another example of cells that assumed a flat, expanded shape with some processes.
Figure 7f shows the same cells as in 7e after staining for GFAP.

Figure 8 shows analysis of LPC cell division on conventional culture dish or after encapsulation in peptide hydrogel.
Figure 8a is a phase contrast micrograph showing cells immediately after encapsulation. (400X magnification).

Figure 8b is a phase contrast micrograph showing cells 24 hours after encapsulation. (400X magnification).

Figure 8c is a phase contrast micrograph showing cells 48 hours after encapsulation illustrating adoption of a spheroid morphology (approximately 5-6 cells, 400X magnification).

Figure 8d is a phase contrast micrograph showing cells 96 hours after encapsulation illustrating a spheroid containing approximately 10-14 cells. (400X magnification).

Figure 8e shows a phase contrast micrograph of a control colony grown on a conventional tissue culture dish for 48 hours. (200X magnification).

Figure 8f shows the same colony as in 8e immunostained for BrdU. (200X magnification).

Figure 8g shows a phase contrast micrograph of a spheroid colony after 96 hours of growth encapsulated in peptide hydrogel. (200X magnification).

Figure 8h shows the same colony as in 8g immunostained for BrdU. (200X magnification).

Figure 9 shows phenotypic analysis of LPC cells during exponential growth on conventional culture dish either after isolation from regular culture conditions or after isolation from peptide hydrogel culture.

Figure 9a is a phase contrast micrograph of a control colony.

Figure 9b shows the same cells as in 9a after staining for C/EPBα.

Figure 9c is a phase contrast micrograph of a spheroid containing cells isolated from peptide hydrogel.

Figure 9d shows the same cells as in 9c after staining for C/EPBα.

Figure 9e is a phase contrast micrograph of a control colony.

Figure 9f shows the same cells as in 9e after staining for albumin.

Figure 9g is a phase contrast micrograph of a spheroid containing cells isolated from peptide hydrogel.

Figure 9h shows the same cells as in 9g after staining for albumin.

Figure 9i is a phase contrast micrograph of a control colony.

Figure 9j shows the same cells as in 9g after staining for CYP1A1/1A2.

Figure 9k is a phase contrast micrograph of a spheroid containing cells isolated from peptide hydrogel.

Figure 9l shows the same cells as in 9g after staining for CYP1A1/1A2.

Figure 10 shows induction of CYP1A1 and CYP1A2 activities by 3-methylcholanthrene (3-MC) in LPC control cultures and hydrogel derived LPC spheroid colonies. Units of activity are expressed as picomoles/cell/hour and the data correspond to the average value from triplicates and their standard deviation (SD). In each panel, CYP activity is presented with circles in non induced (-3-MC) cultures, and with squares in induced (+3-MC) cultures. The arrow in each panel indicates the time at which the inducer 3-MC was added to the cultures.

Figure 10a shows CYP1A1 activity measured in LPC control cultures.

Figure 10b shows CYP1A2 activity measured in hydrogel derived LPC spheroid colonies.

Figure 10c shows CYP1A1 activity measured in LPC control cultures.

Figure 10d shows CYP1A2 activity measured in hydrogel derived LPC spheroid colonies.

Figure 11 compares LPC spheroids growing in peptide hydrogels in the absence or presence of ECM components.

Figures 11a and 11b show phase contrast micrographs of LPC spheroids growing in peptide hydrogels in the absence of ECM components and growth factors. Bars represent 100 μm in length.

Figures 11c and 11d show phase contrast micrographs of LPC spheroids formed by cells grown in the presence of laminin, EGF, and FGF-2. Bars represent 100 μm in length.

Figure 12 shows immunofluorescence staining for insulin in hydrogel derived LPC colonies cultured under various conditions.

Figure 12a is a phase contrast micrograph of a control colony obtained after 8 days of culture in peptide hydrogel followed by overnight culture on a regular plastic culture dish.

Figure 12b shows the same optical layer as Figure 12a after staining for insulin.

Figure 12c is a phase contrast micrograph of a colony obtained after 8 days of culture in peptide hydrogel with 50 μg/ml fibronectin and 0.8 ng/ml EGF followed by overnight culture on a regular plastic culture dish.

Figure 12d shows the same optical layer as Figure 12c after staining for insulin.

Figure 12e is a phase contrast micrograph of a colony obtained after 8 days of culture in peptide hydrogel with 50 μg/ml laminin-1, 0.8 ng/ml EGF, and 0.5 ng/ml FGF-2 followed by overnight culture on a regular plastic culture dish.

Figure 12f shows the same optical layer as in Figure 12e after staining for insulin.

Figure 13 is a chart comparing insulin release by hydrogel-derived spheroids cultured according to a pancreatic differentiation protocol following stimulation by various stimulating conditions.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

I. Overview

Stem cells have been defined as cells that are able to both self-renew, i.e., to divide and create additional stem cells, and also to give rise to cells that can undergo differentiation along a specified pathway or pathways (See, e.g., Fuchs, E. and Segre, J., "Stem Cells: A New Lease on Life", *Cell*, 100, 143-155, 2000; Weissman, I., "Stem Cells: Units of Development, Units of Regeneration, and Units in Evolution", *Cell*, 100, 157-168, 2000). Thus stem cells themselves are undifferentiated but are able to generate one or more specialized cell types with specific functions in the body. Differentiation refers to a qualitative change in cellular phenotype that is the consequence of the synthesis of new gene products (Loeffler, M. and Potten, C., "Stem cells and cellular pedigrees - a conceptual introduction", in *Stem Cells*, Potten, C. (ed.), Academic Press, San Diego, 1997).

Differentiation or transdifferentiation may be recognized by a morphological change in the cell or by detecting changes in enzyme activity or protein composition. Proteins (including enzymes) and the mRNAs that encode such proteins that may be used to characterize a particular differentiation pathway or state are referred to herein as markers. Such markers may not be unique to a particular differentiated

cell type but may be found in a variety of differentiated cell types. For example, cytochrome P450 enzymes are produced by mature hepatocytes and are thus markers for hepatocyte differentiation. However, some of these enzymes are also produced by cells in the intestine and are thus also markers for these intestinal cells. One of ordinary skill in the art will readily be able to select an appropriate marker or combination of markers that are sufficient to identify a cell as being of a particular differentiated cell type.

Two different types of stem cells have been recognized. Embryonic stem cells, present in the embryonic blastocyst, are sufficiently undifferentiated that they are able to give rise to any cell type. Somatic stem cells are found in specific adult tissues, where they are thought to be able to divide indefinitely. Until recently it was widely believed that somatic stem cells possessed a restricted *in vivo* differentiation capability, e.g., that they were only able to give rise to differentiated cells characteristic of the tissues in which they were found. Recent results, however, have suggested that somatic stem cells may have a much broader range of differentiation possibilities than previously thought. For example, neural stem cells appear able to give rise to hematopoietic cells *in vivo* (Bjornson, C., *et al.*, *Science*, 283:534-537), while hematopoietic stem cells can differentiate into hepatocytes (Lagasse, E., *et al.*, "Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. *Nature Medicine*, 6:1229-1234, 2000). These and other similar results, based largely on *in vivo* studies, have highlighted the importance of the phenomenon of cellular plasticity.

As used herein, the term "progenitor cell" refers to a cell that is not fully differentiated but that has the capacity to give rise to a daughter cell or cells that are able to do so. Thus a stem cell is one type of progenitor cell. As is known in the art, a stem cell is generally considered to have broader differentiation potential than other types of progenitor cells. Stem cells are generally considered to have the ability to self-renew. The term "progenitor cell" also includes cells that may have undertaken one or more steps along a differentiation pathway, e.g., that express one or more differentiation markers. The terms "precursor cell" and "progenitor cell" are used interchangeably herein. The term "stem cell" is understood to refer to either adult stem cells, e.g., liver stem cells, or embryonic stem cells.

In order to more fully exploit the potential of progenitor cells and stem cells it is desirable to be able to control and manipulate their differentiation. In particular, it would be desirable to control cellular transdifferentiation. As used herein, the term "transdifferentiation" refers to (1) the capacity of a progenitor cell isolated from a particular body tissue or organ, either to differentiate into cells of a type normally found in a different body tissue or organ or to give rise to a daughter cell or cells that are able to do so or (2) the capacity of a cell that exhibits a cellular morphology characteristic of a particular cell lineage or expresses one or more differentiation markers characteristic of a particular cell lineage to alter its developmental fate and to adopt an alternate differentiation pathway. These alternative definitions are not mutually exclusive and will frequently overlap. Altering the differentiation or transdifferentiation potential of a cell may be referred to as cellular reprogramming, which may also include inducing a cell that has exited the cell cycle, e.g., a post-mitotic cell, to re-enter the cell cycle. Agents that enhance or stimulate transdifferentiation, and/or cell cycle reentry may be referred to as reprogramming agents.

The present invention provides compositions and methods for manipulating the extracellular environment of a progenitor cell or stem cell including compositions and methods for enhancing differentiation and transdifferentiation. Such compositions and methods include growing cells under physical conditions that render the cells permissive for instruction along particular differentiation pathways. The physical conditions can include, in particular, encapsulating progenitor cells within three-dimensional peptide hydrogels. The instruction can comprise exposing the cells to one or more growth or differentiation factors and/or one or more extracellular matrix (ECM) components. In preferred embodiments of the invention at least part of the exposure occurs while the cells are cultured within a three-dimensional nanoscale material such as a peptide hydrogel as described herein. The compositions and methods of the present invention allow harnessing of the inherent regenerative capacity of progenitor cells in new and useful ways.

The regenerative capacity of the body is particularly striking in the case of certain organs such as the liver, which is well-known to possess extensive capacity to regenerate after insults ranging from partial hepatectomy to toxin-induced injury.

Unlike other regenerating tissues such as bone marrow and skin, liver regeneration is thought not to depend on a small group of progenitor cells. Instead, regeneration occurs through the division of mature cell populations including hepatocytes (the main functional cells of the liver, which synthesize a wide range of proteins including enzymes that serve key roles in metabolism of exogenous and endogenous compounds); biliary epithelial cells (which line biliary ducts); endothelial cells; Kupffer cells (macrophages); and stellate cells, also referred to as Ito cells (which are located under liver sinusoids, surround hepatocytes with long processes, and secrete extracellular matrix proteins and growth factors). Liver regeneration is extensively reviewed in Michalopoulos, G. and DeFrances, M., "Liver Regeneration", *Science*, 276, 60-66, 1997.

Nevertheless, cells with stem cell or precursor cell properties do appear in large numbers when mature hepatocytes are prevented from proliferating. These cells are able to give rise *in vivo* to some or all of the mature cell types found in the liver. While it is possible to culture liver-derived cells *in vitro* success in maintaining long-term cultures of fully differentiated, functional hepatocytes has been limited. Cultured hepatocytes tend to lose certain aspects of the mature hepatocyte phenotype. This feature has hampered the development of cell culture systems in which to study hepatotropic viruses such as hepatitis B virus, which replicate in mature hepatocytes. In addition, it limits the utility of liver-derived cells in medical applications such as tissue engineering (e.g., artificial livers), cell transplantation therapies, and gene therapy.

Unlike the central nervous system, the liver represents an organ that is readily accessible for the removal of tissue. The inherent regenerative capacity of the liver and the fact that even a fraction of the normal liver mass is able to fulfill the body's needs suggest that the liver can serve as a convenient source of cells for *in vitro* culture and medical applications, not necessarily limited to the treatment of liver-related conditions.

The present invention represents a convergence of research in the fields of stem and progenitor cell biology and technology and research in the field of biological materials. The development of new biological materials, particularly biologically compatible materials that serve as permissive substrates for cell growth,

differentiation, and biological function has broad implications for advancing medical technology and for understanding basic biological characteristics of cells. The inventors have previously described a class of biomaterials that are made through self-assembly of ionic self-complementary peptides (Zhang, S., *et al.*, *Proc. Natl. Acad. Sci. USA*, 90, 3334-3338, 1993; Zhang, S., *et al., Biomaterials*, 16, 1385-1393, 1995; U.S. Patent Numbers 5,955,343 and 5,670,483). These materials are hydrogels, which in certain embodiments contain approximately 99% or greater water content. They self-assemble into membranes or three-dimensional structures upon exposure to a sufficient concentration of ions. The sequences, characteristics, and properties of the peptides and the structures formed by them upon self-assembly are further discussed in the next section.

The inventors have shown that these peptide structures are able to support cell attachment, viability, and growth when cells are cultured on the surface of the structure. In addition, the structures (also referred to herein as scaffolds) are able to serve as substrates for neurite outgrowth and synapse formation when neurons are grown on their surface (Holmes, T., *et al.*, *Proc. Natl. Acad. Sci.*, 97(12), 2000). In addition, inventors have shown that it is possible to encapsulate cells within the peptide hydrogels, thus placing the cells in a three-dimensional arrangement within the peptide structure, and that the cells maintain viability and function when so encapsulated (see pending U.S. Patent Application Serial No. 09/778200, filed February 6, 2001, Entitled "Peptide Scaffold Encapsulation Of Tissue Cells And Uses Thereof"). Inventors showed, for example, that chondrocytes encapsulated within peptide structures are able to synthesize extracellular matrix components.

In order to further explore the phenomenon of cellular plasticity and to develop ways to modulate and control the division, differentiation, and transdifferentiation of progenitor cells *in vitro*, the inventors decided to examine the effects of encapsulation within peptide hydrogel structures on these parameters. Inventors have discovered now discovered new and unexpected properties of the peptide structures in that they are able to promote differentiation and transdifferentiation of encapsulated cells. The peptide structures are able render progenitor cells permissive for instruction by differentiation-enhancing factors to

which they would not respond under standard culture conditions or to alter the differentiation response of cells to such factors.

Somatic cells (LPCs) were isolated from liver (see Example 1) and cultured in three dimensional peptide structures (peptide hydrogels) in various media and in the presence of different growth factor(s). Because of the cellular plasticity exhibited by these cells, they are referred to as "liver progenitor cells" (LPCs). The name LPC was chosen to indicate the tissue of origin, while not restricting expectations for their differentiation and/or self-renewal potential. The peptide hydrogel structures are described in further detail below.

As presented in more detail in the Examples, the peptide hydrogel provided an environment that altered the phenotypes and differentiation pathways adopted by cells cultured therein. For example, when cultured on traditional plastic culture dishes, rat liver progenitor cells maintain a uniform, flat morphology and do not differentiate well into functional hepatocytes. Such cells fail to express significant amounts of cytochromes P4501A1 (CYP1A1) or P4501A2 (CYP1A2), enzymes produced by mature hepatocytes. In contrast, rat liver progenitor cells cultured in a three-dimensional peptide hydrogel divide to form spheroidal clusters, reminiscent of the behavior of mature hepatocytes under certain culture conditions. Furthermore, a portion of cells maintained in the hydrogels express CYP1A1 and/or CYP1A2 and display CYP1A1 and CYP1A2 enzymatic activity such as CYP1A1 and CYP1A2-dependent *O*-dealkylation activities on a resorufin alkyl ether substrate (7-ethoxyresorufin and 7-methoxyresofurin, respectively), and ability to catalyze 3-*N*-demethylation of the caffeine (1,3,7-trimethylxanthine) to produce paraxanthine (1,7-dimethylxanthine) (a CYP1A2-specific metabolic activity). In addition, both CYP activities were induced by the polycyclic aromatic hydrocarbon 3-methylcholanthrene (3-MC) in the hydrogel derived spheroid colonies but not in control cultures, as observed in mature hepatocytes. This result suggests the presence of functional aromatic hydrocarbon (Ah) receptors on the hydrogel-derived spheroid colony cell membranes, further confirming the hepatocyte-like phenotype.

In hepatocytes the ligand-induced transcription factor Ah receptor (AHR) regulates a biphasic pleiotropic response to a variety of environmental contaminants with polycyclic aromatic structures such as 3-MC and benzo[a]pyrene (Burbach,

K.M., Poland, A. & Bradfield, C.A. "Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor", *Proc. Natl. Acad. Sci. USA* 89, 8185-8189 (1992)). The response includes an increase in expression of xenobiotic metabolizing enzymes including cytochrome P450 1A1, P450 1A2, and P450 1B1-dependent monooxygenases (Jones, P.B.C., Galeazzi, D.R., Fisher, J.M. & Whitlock, J.P., Jr. "Control of cytochrome P1-450 gene expression by dioxin", *Science* 277, 1499-1502 (1985)). The finding that CYP1A1 and CYP1A2 activities, which play a major role in hepatic biotransformation and detoxification of a wide range of xenobiotics, can be significantly induced by 3-methylcholanthrene exposure in LPCs cultured in peptide hydrogels indicates the potential application of the system for a variety of applications including screening for toxicity of potential pharmaceutical agents and other compounds. Table 6 presents a summary of experiments and data confirming the hepatocyte-like phenotype of LPCs cultured in peptide hydrogels. Table 6 presents data on expression of fetal and mature hepatocyte and/or oval cell or biliary epithelial cell markers, hepatocyte functional activity, and morphology.

To further investigate the differentiation phenomena, cells in peptide hydrogels were switched to a defined hepatocyte growth medium. Surprisingly, by 24 hours after the medium was changed, a considerable proportion of the cells acquired a dramatic change in cellular morphology, exhibiting very elongated cell bodies with rudimentary processes. The phenotype of these cells resembled that of neuronal lineages. Staining for various markers characteristic of neuronal lineage cells further indicated that the cells were developing along a non-hepatocyte pathway. Results were consistent with a conclusion that these cells possessed features of neuronal precursors. Acquisition of neuronal-like phenotypes was dependent on the presence of various growth factors (EGF or EGF/NGF) in the culture medium and did not occur when cells were cultured on plates in identical medium. Thus within the hydrogel liver progenitor cells are being instructed to divide and differentiate both in hepatocyte-like pathways and in pathways distinct from those assumed by normal hepatocytes. Thus encapsulation within the peptide hydrogel structures rendered cells permissive for instruction by various growth factors. In the presence of either EGF or EGF/NGF, the liver progenitor cells

cultured within the peptide hydrogel structure exhibited the ability to differentiate along a hepatocyte-like lineage and to transdifferentiate along a neuronal lineage. Cells grown in soft agar did not proliferate or exhibit either hepatocyte-like or neuronal-like phenotypes, suggesting that the mere arrangement of the cells in three dimensions is not sufficient to allow these effects.

Liver and ventral pancreas appear to arise from the same cell population within the embryonic endoderm (Sell, S. and Ilic, Z., *Liver Stem Cells*, Chapman and Hall, New York, 1997). Studies have demonstrated the possible existence of a bipotential precursor population for pancreas and liver during mammalian embryonic development (Deutsch, G. *et al.*, *Development* 128, 871-881, 2001). While not wishing to be bound by any theory, the inventors hypothesized that since liver and pancreas cells may originate from a common lineage, adult liver stem cells could be stimulated to differentiate into pancreatic lineage cells of one or more types. Pancreatic tissue comprises both exocrine and endocrine cell types. It is believed that all endocrine cells of the pancreatic islets of Langerhans, including glucagon-producing α cells, insulin-producing β cells, pancreatic polypeptide producing γ cells, and somatostatin producing δ cells originate from the same ductal epithelial stem cells through sequential differentiation. Pancreatic exocrine cells secrete a number of digestive enzymes including trypsin, chymotrypsin, elastase, lipases, amylase, etc., in addition to secreting bicarbonate. Pancreatic cell types can be identified by their expression of genes encoding these polypeptide products and also by their expression of a variety of other markers.

Several studies have investigated the effects of various growth factors and extracellular matrix proteins on the differentiation of embryonic stem cells into pancreatic β-cells (Soria, B., *Differentiation* 68, 205-219, 2001). The inventors selected epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), and the extracellular matrix proteins laminin and fibronectin to initially characterize the effect of growth factors and extracellular (ECM) matrix components alone or in combination on the differentiation of LPCs cultured in the hydrogel context into cells characteristic of the pancreas, e.g., pancreatic-like lineage cells. As described in more detail in Example 8, LPCs cultured in hydrogels in the presence of various combinations of growth factors and ECM components transdifferentiated into

pancreatic lineage-like cells. In particular, LPCs cultured in peptide hydrogels containing laminin in the presence of EGF, FGF-2, or both transdifferentiated into structures resembling pancreatic islets containing β-like cells capable of producing insulin and secreting insulin in response to various stimuli. In addition, LPCs cultured in peptide hydrogels containing fibronectin in the presence of EGF transdifferentiated in a similar manner.

The data presented herein indicates that a three-dimensional nanoscale environment comprising a peptide hydrogel is able to support the growth and differentiation of cells having properties of liver cell precursors, liver stem cells, hepatocytes, and oval cells. In addition, the hydrogel is able to support the growth, trans-differentiation, and differentiation of cells having properties of neural lineage cells. Furthermore, the hydrogel environment is able to support the growth and functional activity of cells having properties of endocrine cells such as β-like insulin producing pancreatic cells. Cell types such as these exist in a wide variety of three-dimensional environments within the body. Thus the hydrogel is able to support the growth, trans-differentiation, and/or differentiation of a wide range of cell types, e.g., cell types that may exist in a variety of different three-dimensional environments within the body. In particular, the hydrogel provides an environment conducive to the differentiation or transdifferentiation of liver progenitor cells into a variety of different cell types. Such cell types include cells of liver tissue origin including cells that functionally and morphologically resemble mature hepatocytes, cells of neural tissue origin, and cells of pancreatic origin including insulin producing β-like cells.

The invention thus provides a composition comprising a peptide hydrogel structure encapsulating progenitor cells and a differentiation or transdifferentiation enhancing factor such as an ECM component or growth factor, which may be present or added to medium in which the structure is cultured. In certain embodiments of the invention the progenitor cell is a liver progenitor cell, i.e., a cell that does not express a fully differentiated liver-specific phenotype but that has the capacity, under appropriate conditions, to give rise to cells that assume a liver-specific cellular morphology and/or express a marker characteristic of a liver-specific cell. In certain embodiments of the invention the liver progenitor cell is

isolated from the liver. In certain other embodiments of the invention the liver progenitor cell may be isolated from another organ, e.g., from the bone marrow.

The invention further provides methods for enhancing cellular differentiation and transdifferentiation comprising encapsulating a progenitor cell within a peptide hydrogel structure and culturing the encapsulated cells in the presence of one or more differentiation or transdifferentiation enhancing factors such as a growth factor or ECM component for a time sufficient to allow differentiation or transdifferentiation to occur. In certain embodiments of the invention the progenitor cell is a liver progenitor cell. The process of differentiation or transdifferentiation may occur within the cell or within progeny or descendants of the cell. The differentiation may be along a liver-specific lineage pathway, e.g., towards a hepatocyte-like phenotype. The transdifferentiation may be along a neuronal-like pathway, e.g., towards a neuronal precursor, a neuron, or a glial cell. The transdifferentiation may be along a pancreatic-like lineage pathway, e.g., towards a pancreatic endocrine cell such as a β cell or an exocrine cell. Both transdifferentiation and differentiation may occur among the progeny of the encapsulated cells, and different cells within the peptide structure may transdifferentiate along different lineage pathways.

While inventors have not yet identified the precise mechanism by which the peptide structures exert their effect, and while not wishing to be bound by any theory, inventors propose a number of possibilities that may be systematically explored and parameters that may be varied in order to refine and expand upon the discoveries and inventions described herein. For example, the peptide sequence, length, and concentration may be varied, which may in turn affect the stiffness, oxygen tension, fraction of cell surface in contact with the gel, and/or growth factor gradients within the structure. The particular growth factor(s) and ECM components may be varied as may their concentrations. All such improvements and refinements are within the scope of the invention.

The sections below address various aspects of the invention including peptide structures, cells that may be used in the practice of the invention, methods of encapsulating cells within peptide structures of the invention, and inventive culture techniques that may be employed in the context of cells cultured in or on peptide

structures, in order to achieve various effects on cell division and phenotype. Methods for monitoring the effects of the peptide structures and culture conditions on cell division and phenotype are also presented.

Among the embodiments of the invention are (i) an *in vitro* culture system for studying stem and progenitor cells and their differentiation and transdifferentiation properties; (ii) an *in vitro* culture system for growing hepatocyte-like cells and/or mature hepatocytes, which may be used, for example, to propagate hepatotropic viruses; (iii) a system for controlling and manipulating cell differentiation and transdifferentiation *in vitro*, from which cells can be extracted and then either maintained *in vitro* or administered to a subject; (iv) an *in vitro* culture system for testing the toxicity of compounds and evaluating their metabolism, biotransformation, etc., (v) a system for controlling and manipulating cell differentiation and transdifferentiation *in vitro* within a structure that is to be implanted within a subject, e.g., for treating a disorder or disease. Various other embodiments of the invention such as cell culture kits and assay systems are also described.

II. Peptide Structures and Methods of Encapsulating Cells

Inventors have discovered a class of certain peptides consisting of alternating hydrophilic and hydrophobic amino acids that are capable of self-assembling to form an exceedingly stable beta-sheet macroscopic structure in the presence of electrolytes, such as monovalent cations. The peptides are complementary and structurally compatible. These peptides and their properties are described in U.S. Patent Numbers 5,955,343 and 5,670,483 and in co-pending U.S. Patent Application Serial No. 09/778200, filed February 6, 2001, entitled "Peptide Scaffold Encapsulation Of Tissue Cells And Uses Thereof". For example, NaCl at a concentration of between 5 mM and 5 M induces the assembly of macroscopic structures within a few minutes. Lower concentrations of NaCl may also induce assembly but at a slower rate. The side-chains of the peptides in the structure partition into two faces, a polar face with charged ionic side chains and a nonpolar face with alanines or other hydrophobic groups. These ionic side chains are self-complementary to one another in that the positively charged and negatively

charged amino acid residues can form complementary ionic pairs. These peptides are therefore called ionic, self-complementary peptides, or Type I self-assembling peptides. If the ionic residues alternate with one positively and one negatively charged residue(- + - + -+ - +), the peptides are described as "modulus I;" if the ionic residues alternate with two positively and two negatively charged residues (- - + + - - ++), the peptides are described as "modulus II."

Many modulus I and II self-complementary peptides with identical compositions and length, such as EAK16, KAE16, RAD16, RAE16, and KAD16, have been analyzed previously (Table 1). Modulus IV ionic self-complementary peptides containing 16 amino acids; such as EAK16-IV, KAE16-IV, DAR16-IV and RAD16-IV; has also been studied. If the charged residues in these self-assembling peptides are substituted (*i. e.*, the positive charged lysines are replaced by positively charged arginines and the negatively charged glutamates are replaced by negatively charged aspartates), there are essentially no significant effects on the self-assembly process. However, if the positively charged resides, lysine and arginine are replaced by negatively charged residues, aspartate and glutamate, the peptides can no longer undergo self-assembly to form macroscopic structures; however, they can still form a beta-sheet structure in the presence of salt. Other hydrophilic residues, such as asparagine and glutamine, that form hydrogen-bonds may be incorporated into the peptides instead of or in addition to charged residues. If the alanines in the peptides are changed to more hydrophobic residues, such as leucine, isoleucine, phenylalanine or tyrosine, these peptides have a greater tendency to self-assemble and form peptide matrices with enhanced strength. Some peptides that have similar compositions and lengths as these aforementioned peptides form alpha-helices and random-coils rather than beta-sheets and do not form macroscopic structures. Thus, in addition to self-complementarity, other factors are likely to be important for the formation of macroscopic structures, such as the peptide length, the degree of intermolecular interaction, and the ability to form staggered arrays.

Table 1. Representative Self-Assembling Peptides

Name	Sequence (n-->c)	Modulus
RAD16-I	n-RADARADARADARADA-c	I
RGDA16-I	n-RADARGDARADARGDA-c	I
RADA8-I	n-RADARADA-c	I
RAD16-II	n-RARADADARARADADA-c	II
RAD8-II	n-RARADADA-c	II
EAKA16-I	n-AEAKAEAKAEAKAEAK-c	I
EAKA8-I	n-AEAKAEAK-c	I
RAEA16-I	n-RAEARAEARAEARAEA-c	I
RAEA8-I	n-RAEARAEA-c	I
KADA16-I	n-KADAKADAKADAKADA-c	I
KADA8-I	n-KADAKADA-c	I
EAH16-II	n-AEAEAHAHAEAEAHAH-c	II
EAH8-II	n-AEAEAHAH-c	II
EFK16-II	n-FEFEFKFKFEFEFKFK-c	II
EFK8-II	n-FEFKFEFK-c	I
ELK16-II	n-LELELKLKLELELKLK-c	II
ELK8-II	n-LELELKLK-c	II
EAK16-II	n-AEAEAKAKAEAEAKAK-c	II
EAK12	n-AEAEAEAEAKAK-c	IV/II
EAK8-II	n-AEAEAKAK-c	II
KAE16-IV	n-KAKAKAKAEAEAEAEA-c	IV
EAK16-IV	n-AEAEAEAEAKAKAKAK-c	IV
RAD16-IV	n-RARARARADADADADA-c	IV
DAR16-IV	n-ADADADADARARARAR-c	IV
DAR16-IV*	n-DADADADARARARARA-c	IV
DAR32-IV	n-(ADADADADARARARAR)-c	IV
EHK16	n-HEHEHKHKHEHEHKHK-c	N/A

EHK8-I	n-HEHEHKHK-c	N/A
VE20*	n-VEVEVEVEVEVEVEVEVEVE-c	N/A
RF20*	n-RFRFRFRFRFRFRFRFRFRF-c	N/A

N/A denotes not applicable

* These peptides form a β-sheet when incubated in a solution containing NaCl, however they have not been observed to self-assemble to form macroscopic scaffolds.

Other self-assembling peptides may be generated by changing the amino acid sequence of any self-assembling peptide by a single amino acid residue or by multiple amino acid residues. Additionally, the incorporation of specific cell recognition ligands, such as RGD and RAD, into the peptide scaffold may promote the proliferation of the encapsulated cells. *In vivo* these ligands may also attract cells from outside a scaffold to the scaffold, where they may invade the scaffold or otherwise interact with the encapsulated cells. To increase the mechanical strength of the structures, cysteines may be incorporated into the peptides to allow the formation of disulfide bonds, or residues with aromatic rings may be incorporated and cross-linked by exposure to UV light. The *in vivo* half-life of the scaffolds may also be modulated by the incorporation of protease cleavage sites into the scaffold, allowing the scaffold to be enzymatically degraded. Combinations of any of the above alterations may also be made to the same peptide structure.

Peptides capable of being cross-linked may be synthesized using standard f-moc chemistry and purified using high pressure liquid chromatography. The formation of a peptide structure may be initiated by the addition of electrolytes as described herein. Hydrophobic residues with aromatic side chains may be crossed-linked by exposure to UV irradiation. The extent of the cross-linking may be precisely controlled by the predetermined length of exposure to UV light and the predetermined peptide concentration. The extent of cross-linking may be determined by light scattering, gel filtration, or scanning electron microscopy using standard methods. Furthermore, the extent of cross-linking may also be examined by HPLC or mass spectrometry analysis of the scaffold after digestion with a

protease, such as matrix metalloproteases. The material strength of the scaffold may be determined before and after cross-linking.

Aggrecan processing sites may be added to the amino- or carboxy-terminus of the peptides or between the amino-and carboxy- termini. Likewise, other matrix metalloproteases (MMPs) cleavage sites, such as those for collagenases, may be introduced in the same manner. Peptide structures formed from these peptides, alone or in combination with peptides capable of being cross-linked, may be exposed to various protease for various lengths of time and at various protease and scaffold concentrations. The rate of degradation of the scaffolds may be determined by HPLC, mass spectrometry, or NMR analysis of the digested peptides released into the supernatantat various time points. Alternatively, if radiolabeled peptides are used for scaffold formation, the amount of radiolabeled peptides released into the supernatant maybe measured by scintillation counting. Cross-linking and cleavage studies are described further in pending U.S. Patent Application Serial No. 09/778200, filed February 6, 2001, Entitled "Peptide Scaffold Encapsulation Of Tissue Cells And Uses Thereof".

If desired, the peptide scaffolds formed from any of the above peptides may be characterized using various biophysical and optical instrumentation, such as circular dichroism (CD), dynamic light scattering, Fourier transform infrared (FTIR), atomic force microscopy (ATM), scanning electron microscopy (SEM),and transmission electron microscopy (TEM). For example, biophysical methods may be used to determine the degree of beta-sheet secondary structure in the peptide structure. Additionally, filament and pore size, fiber diameter, length, elasticity, and volume fraction may be determined using quantitative image analysis of scanning and transmission electron microscopy. The structures may also be examined using several standard mechanical testing techniques to measure the extent of swelling, the effect of pH and electrolyte concentration on structure formation, the level of hydration under various conditions, and the tensile strength.

The peptide hydrogels described herein are significantly different from other biopolymer-based biomaterials for several reasons. Most biomaterials have fiber sizes diameters generally in the 10-20 micron range (microscale), similar in scale to the size of many types of cells. When grown in an environment comprising such

microscale fibers, cells attach to the microfiber with a curvature. Cells grown in a typical biopolymer-based biomaterial are also less hydrated than when grown in the nanoscale environment created by the self-assembling peptide hydrogels described herein. In certain embodiments of the invention the self-assembling peptides described herein are approximately 5 nm in length and approximately 1 nm in diameter. Such peptides undergo self-assembly to form nanofibers (e.g., fibers having a diameter of approximately 10-20 nm). While not wishing to be bound by any theory, inventors suggest that in such an environment cells truly experience three dimensional spatial enclosures on a scale that is relevant to cellular dimensions. The peptides undergo self-assembly to form nanofibers that are highly hydrated (e.g., up to 99.5-99.9%% (1-5 mg/ml) water). Because the hydrogel has such an extremely high water content, cells can freely migrate and form intercellular contacts and structures such as the spheroids described herein. Such environment also permits diffusion of small molecules including proteins and signaling molecule exchanges.

Peptide structures may be generated in a variety of shapes and geometries by forming the structure within an appropriately shaped mold. Where the peptide structure or scaffold is to be implanted into the body, the shape may be selected based upon the intended implantation site, for example.

To encapsulate cells within a peptide structure, peptides and living cells may be incubated in an aqueous solution having an iso-osmotic solute (i.e., a solute at an appropriate concentration to support cell viability), under conditions that do not allow the peptides to substantially self-assemble. In certain embodiments of the invention the solution contains less than 10, 5, 1, or 0.1 mM electrolyte or is substantially free of electrolyte. Sufficient electrolyte is added to the solution to initiate self-assembly of the peptides into a beta-sheet macroscopic structure ,whereby the cells are encapsulated by the formation of the macroscopic structure. The encapsulated cells are present in the macroscopic structure in a three-dimensional arrangement. In certain embodiments of the invention the concentration of the added electrolyte is at least 5, 10, 20, or 50 mM. Suitable electrolytes include, but are not limited to, Li^+, Na^+, K^+, and Cs^+. In some embodiments, the concentration of the iso-osmotic solute is at least 50, 150, or 300

mM. In other embodiments, the concentration of the iso-osmotic solute is contained in one of the following ranges 200 to 250 mM, 250 to 270 mM, 270 to 300 mM, 300 to 400 mM, 400 to 500 mM, 500 to 600 mM, 600 to 700 mM, 700 to 800 mM, or 800 to 900 mM, inclusive. Suitable iso-osmotic solutes include, but are not limited to, carbohydrates, such as sucrose, mannitol, etc. Other iso-osmotic solutes, preferably non-toxic to cells at the concentration used, may be employed.

In order to form a macroscopic structure of predetermined shape or volume, peptides and living cells may be incubated in an aqueous solution having an iso-osmotic solute, under conditions that do not allow the peptides to substantially self-assemble. In certain embodiments of the invention the solution contains less than 10, 5, 1, or 0.1 mM electrolyte or is substantially free of electrolytes. The solution is contained in a pre-shaped mold dimensioned to determine the volume or shape of the macroscopic structure. Sufficient electrolyte is added to the solution to initiate self-assembly of the peptides into a beta-sheet macroscopic structure, whereby the cells are encapsulated by the formation of the macroscopic structure. The encapsulated cells are present in the structure in a three-dimensional arrangement. The concentration of the added electrolyte may be at least 5, 10, 20, or 50 mM. Suitable electrolytes include Li^+, Na^+, K^+, and Cs^+. In one embodiment, the concentration of the iso-osmotic solute is at least 50, 150, or 300 mM. In another embodiment, the concentration of the iso-osmotic solute is contained in one of the following ranges 200 to 250 mM, 250 to 270 mM, 270 to 300 mM, 300 to 400 mM, 400 to 500 mM, 500 to 600 mM, 600 to 700 mM, 700 to 800 mM, or800 to 900 mM, inclusive. Suitable iso-osmotic solutes include, but are not limited to, carbohydrates such as sucrose, etc.

The peptide structures may be used for regenerating a tissue, and the invention includes methods for such use. The methods includes administering to an animal, such as a mammal (including a human) a macroscopic scaffold structure having amphiphilic peptides and encapsulated living progenitor cells and/or their progeny. The peptides have alternating hydrophobic and hydrophilic amino acids, are complementary and structurally compatible, self-assemble into a beta-sheet macroscopic structure and render the progenitor cells or their progeny permissive for instruction to differentiate and/or transdifferentiate.

The encapsulated cells are present in the macroscopic structure in a three-dimensional arrangement. The density of the cells may be approximately 10^5/ml, between 5 x 10^5/ml and 5 x 10^6/ml, inclusive, between 5 x 10^4/ml and 5 x 10^5/ml, between 5 x 10^5/ml and 5 x 10^6/ml. Other ranges may also be used. Conditions for culturing should be close to physiological conditions. The pH of the culture medium should be close to physiological pH, preferably between pH 6-8, for example about pH 7 to 7.8, in particular pH 7.4. Physiological temperatures range between about 30° C to 40°C. Mammalian cells are preferably cultured at temperatures between about 32° C to about 38°C, e.g., between about 35° C to about 37° C.

Cells may be cultured within the peptide scaffold for any appropriate time, depending upon the cell number and density desired, the proliferation rate of the cells, and the time required for the desired differentation and/or transdifferentiation to occur. These parameters will vary depending upon the particular progenitor cells and purposes for which the invention is to be used. One of ordinary skill in the art will be able to vary these parameters and to observe the effects of doing so, in order to determine the optimal time for maintaining cells in culture within the scaffold. In certain embodiments of the invention the cell are cultured for approximately 3 days, 7 days, 14 days, 21 days, 28 days, 56 days, or 90 days. In certain embodiments of the invention the cells are cultured for between 1 and 3 days inclusive, between 4 and 7 days inclusive, between 8 and 14 days inclusive, between 15 and 21 days inclusive, between 22 and 28 days inclusive, between 29 and 56 days inclusive, or between 57 and 90 days inclusive. Longer or shorter culture periods may also be used.

The peptide scaffold encapsulating cells may be used to treat a variety of tissue defects, including nervous tissue defects, liver defects, pancreatic tissue defects, connective tissue defects, etc. Cell types that may be used are described further below. The peptide hydrogel structure may be implanted into the body, e.g., surgically or using any other type of suitable procedure. Other routes, including oral, percutaneous, intramuscular, intravenous, and subcutaneous may be employed. One of ordinary skill in the art will be able to select an appropriate delivery technique. The macroscopic structure may assemble prior to administration, but in

certain embodiments of the invention the progenitor cells and peptides are mixed *in vitro* and the structure self-assembles after administration and encapsulates the cells *in vivo*. As described above, in certain embodiments of the invention the administered solution contains less than 10, 5, 1.0, or 0.1 mM electrolyte or is substantially free of electrolyte, and the concentration of the iso-osmotic solute is at least 50, 150, or 300 mM. In other embodiments, the concentration of iso-osmotic solute is contained in one of the following ranges 200 to 250 mM, 250 to 270 mM, 270 to 300 mM, 300 to 400 mM, 400 to 500 mM, 500to 600 mM, 600 to 700 mM, 700 to 800 mM, or 800 to 900 mM, inclusive. Suitable iso-osmotic solutes include, but are not limited to, carbohydrates, such as sucrose. In certain embodiments of the invention, the macroscopic scaffold structure is enzymatically degradable. In other embodiments, the macroscopic scaffold is cleavable by a metalloprotease, collagenase, or aggrecanase *in vivo* or *in vitro*.

In certain embodiments of the invention the macroscopic structure further encapsulates a therapeutically active compound or chemoattractant. Examples of such compounds include synthetic organic molecules, naturally occurring organic molecules, nucleic acid molecules, biosynthetic proteins such as chemokines, or modified naturally occurring proteins. In still other embodiments, the macroscopic structure further encapsulates one or more growth enhancing, differentiation-enhancing, or transdifferentiating-enhancing agents, e.g., growth factors, such as epidermal growth factor, nerve growth factor, transforming growth factor-β, platelet-derived growth factor, insulin-like growth factor, acidic fibroblast growth factor, basic fibroblast growth factor, hepatocyte growth factor, brain-derived neurotrophic factor, keratinocyte growth factor, bone morphogenetic protein, or a cartilage-derived growth factor. Combinations of growth factors and/or therapeutic agents or chemoattractants may be used.

In certain embodiments of the invention the macroscopic structure contains one or more extracellular matrix (ECM) components. The extracellular matrix includes proteins that are secreted locally and are widely distributed. The major types of proteins that constitute the ECM include collagens, elastin, fibronectin, and laminin. The dermis contains ground substance, cellular components including

fibroblasts(which synthesize collagen, elastin, and reticulin), histiocytes, endothelial cells, perivascular macrophages and dendritic cells, mast cells, smooth muscle, and cells of peripheral nerves and their end-organ receptors in addition to proteinaceous components. In many tissues proteinaceous components of the ECM consist largely of collagen (Type I collagen) and reticulin (Type III collagen), which form fibers that help to provide tensile strength. Elastic fibers provide for restoration of shape after a deformation. Ground substance, which undergoes constant turnover (synthesis and degradation) consists largely of glycosaminoglycans, e.g., hyaluronic acid, chondroitin sulfate, and dermatan sulfate.

Collagens are a family of highly characteristic fibrous proteins believed to be present in all multicellular animals. They are the most abundant proteins in mammals, constituting approximately one quarter of total protein. A primary feature of all known collagen molecules is their triple-stranded helical structure. See, for example, Miller and Gay, "The Collagens: An Overview and Update," pp. 3-41, *Methods in Enzymology* (ed. Colowick and Kaplan), v. 144 (1987), Academic Press, Inc.

Elastin, present in elastic fibers of certain tissues such as blood vessels and skin, gives such tissues the required ability to recoil after transient stretch. Elastin is the major component of these elastic fibers, where it is present as an extensively cross-linked polypeptide having a particular chemical composition. Approximately one third of the amino acids in elastin are glycine, 10-13 percent are proline, and over 40 percent are other amino acids with hydrophobic side chains. Elastin contains very small amounts of hydrophilic amino acids.

Laminin, a large glycoprotein and a major component of basement membranes, is made by all epithelial cells studied thus far. Laminin consists of three different subunits disulfide-bonded to form an asymmetric cross-linked structure. For a review see Barlow *et al.*, "Molecular Cloning of Laminin," pp. 404-474 in *Methods in Enzymology*, v. 144 (1987) Academic Press, Inc.

Fibronectin is a cell-surface and blood glycoprotein involved in a variety of cell surface phenomena. It occurs as an insoluble form at the cell surface and in connective tissue. Fibronectin is also found in soluble form in plasma. For

reviews, see Ruoshlati *et al.*, "Fibronectin: Purification, Immunochemical Properties, and Biological Activities," pp. 803-831, in *Methods in Enzymology*, *supra*; Hynes *et al.*, "Isolation and Analysis of cDNA and Genomic Clones of Fibronectin and its Receptor," pp. 447-463, in *Methods in Enzymology*, v. 144, Academic Press, Inc. (1987).

ECM molecules are described in Kreis, T. (ed.) Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins, 2nd Ed., Oxford University Press, 1999; Streuli, C., et al., Extracellular Matrix Protocols (Methods in Molecular Biology, v. 139), Totowa, NJ, Humana Press, 2000 and references therein. ECM components serve a variety of functions in the cellular environment. In addition to serving a structural role, they are likely to be involved in cell signalling and cell communication. ECM components influence cellular access to nutrients, metabolites, growth factors, and chemokines and thus exert a significant influence on cell proliferation and differentiation.

Growth-enhancing, differentiation-enhancing, and/or transdifferentiation-enhancing agents may be added to the peptide solution or to the electrolyte solution prior to initiation of self-assembly. In this case the concentration of the agent will likely be substantially uniform within the assembled scaffold. In certain embodiments of the invention the agent is added to media with which the peptide scaffold is incubated after encapsulation of cells. After addition to the media, a portion of the differentiation-enhancing agent enters the peptide scaffold, e.g., through diffusion. This process may create a gradient of the differentiation-enhancing factor. Cells in different regions of the scaffold may exhibit different responses to the agent depending upon the concentration of the agent at the location of the cell.

In certain embodiments of the invention the peptide structure renders the encapsulated cells permissive to instruction by the differentiation-enhancing or transdifferentiation-enhancing agent. In these embodiments of the invention encapsulated progenitor cells and/or their progeny are induced to differentiate and/or transdifferentiate in the presence of the agent. Growth factors are typically used at concentrations ranging between about 1 fg/ml to 1 mg/ml. Frequently growth factors are used at concentrations in the low nanomolar range, e.g., 1 - 10 nM. In certain

embodiments of the invention growth factors are used at concentrations that are not typically used in the prior art or that are not typically found *in vivo* under normal conditions. For example, growth factors may be used at concentrations that are 5 fold greater, 10 fold greater, 20 fold greater, 100 fold greater, etc., than is typically required to produce effects or than typically occurs *in vivo*. Titration experiments can be performed to determine the optimal concentration of a particular differentiation-enhancing agents, such as a growth factor, depending upon the particular growth, differentiating, and/or transdifferentiating effects desired. ECM components will typically be provided at concentrations in the range of μg/ml, e.g., between 5 and 1000 μg/ml, more typically in ranges of approximately 10 to 100 μg/ml. However, higher or lower concentrations can also be used, e.g., in the micromolar or nanomolar range.

In certain embodiments of the invention the peptides that assemble to form a macroscopic structure have a sequence that includes an adhesion site, growth factor binding site, or sequence that provides targeting to a cell, tissue, organ, organ system, or site within an animal. In certain embodiments of the invention peptides forming the macroscopic scaffold contain between 8 and 200 amino acids, 8 to 36 amino acids, or 8 to 16 amino acids, inclusive. In certain embodiments of the invention the concentration of the peptides is between 1 and 10 mg/ml or between 4 and 8 mg/ml, inclusive.

It is contemplated that the methods of the present invention may be used to repair an injury to an organ or other body structure or to form an organ or other body structure. Such organs or body structures include, but are not necessarily limited to, brain, nervous tissue, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, bladder, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, and uterus.

In certain embodiments of the invention progenitor cells and/or their progeny that have proliferated, differentiated, and/or transdifferentiated within the peptide scaffold are extracted from the scaffold. The extraction may be accomplished by any suitable means, including mechanical disruption of the scaffold, enzymatic degradation of the scaffold *in vitro*, etc. In certain embodiments of the invention the

method selected results in extraction of approximately 25%, between 25% and 50% of the cells inclusive, between 51% and 75% of the cells inclusive, or between 76% and 100% of the cells inclusive. Of course methods that result in any convenient range may be selected. The method selected may depend upon the purposes for which the cells are to be used, the number of cells required, etc. In certain embodiments of the invention the viability of the extracted cells is approximately 10% of cells, between 10% and 25% inclusive, between 26% and 50% of cells inclusive, between 51 and 75% of cells, inclusive, or between 76% and 100% of cells inclusive. Of course methods that result in any convenient range may be selected. The method selected may depend upon the purposes for which the cells are to be used, the number of cells required, etc.

The extracted cells may be further cultured *in vitro*, either in a peptide hydrogel structure or in any other culture vessel. The extracted cells may be administered to a subject by any appropriate route, including intravenous, subcutaneous, oral, percutaneous, intramuscular, or surgical. The administered cells may be used to fill or repair a tissue defect or otherwise supplement an organ or body structure. The administered cells may synthesize or otherwise supply a therapeutic agent. For example, the administered cells may supply a protein, e.g., an enzyme, that the individual lacks. The administered cells may be genetically modified and thus used as a means to deliver gene therapy.

By "scaffold" is meant a three-dimensional structure capable of encapsulating cells. The beta-sheet secondary structure of the scaffold may be confirmed using standard circular dichroism to detect an absorbance minimum at approximately 218 nm and a maximum at approximately 195 nm. The scaffold is formed from the self-assembly of peptides that may include L-amino acids, D-amino acids, natural amino acids, non-natural amino acids, or a combination thereof. If L-amino acids are present in the scaffold, degradation of the scaffold produces amino acids which may be reused by the host tissue. It is also contemplated that the peptides may be covalently linked to a compound, such as a chemoattractant or a therapeutically active compound. The peptides may be chemically synthesized or purified from natural or recombinant sources, and the amino- and carboxy-termini of the peptides may be protected or not protected. The peptide scaffold may be formed

from one or more distinct molecular species of peptides which are complementary and structurally compatible with each other. Peptides containing mismatched pairs, such as the repulsive pairing of two similarly charged residues from adjacent peptides, may also form scaffolds if the disruptive force is dominated by stabilizing interactions between the peptides. Scaffolds are also referred to herein as peptide structures, peptide hydrogel structures, peptide gel structures, or hydrogel structures. Although the practice of the transdifferentiation aspects of the invention to date has involved encapsulation of cells within scaffolds, the possibility that cells may become permissive to instruction by differentiation-enhancing agents when grown on the surface of a peptide structure or membrane has not yet been fully explored. Thus the invention also includes growing progenitor cells and their progeny on the surface of peptide hydrogel scaffolds, wherein the peptide scaffold renders the progenitor cells and/or their progeny permissive for instruction by differentiation-enhancing agents.

By "complementary" is meant the capable of forming ionic or hydrogen bonding interactions between hydrophilic residues from adjacent peptides in the scaffold, as illustrated in Fig. 1, each hydrophilic residue in a peptide either hydrogen bonds or ionically pairs with a hydrophilic residue on an adjacent peptide or is exposed to solvent.

By "structurally compatible" is meant capable of maintaining a sufficiently constant intrapeptide distance to allow scaffold formation. In certain embodiments of the invention the variation in the intrapeptide distance is less than 4, 3, 2, or 1 angstroms. It is also contemplated that larger variations in the intrapeptide distance may not prevent scaffold formation if sufficient stabilizing forces are present. This distance may be calculated based on molecular modeling or based on a simplified procedure that has been previously reported (U.S. Patent Number 5,670,483). In this method, the intrapeptide distance is calculated by taking the sum of the number of unbranched atoms on the side-chains of each amino acid in a pair. For example, the intrapeptide distance for a lysine-glutamic acid ionic pair is 5+4=9 atoms, and the distance for a glutamine-glutamine hydrogen bonding pair is 4+4=8 atoms. Using a conversion factor of 3 angstroms per atom, the variation in the intrapeptide distance of peptides having lysine-glutamic acid pairs and glutamine-glutamine pairs (*e.g.*, 9

versus 8 atoms) is 3 angstroms.

By "substantially uniformly distributed" is meant that immediately after scaffold formation at least 50, 60, 70, 80, 90, or 100% of the cells encapsulated by the scaffold are separated from each other by distances that vary by less than 500, 100, 50, 20, 10, or 1 μM.

By "iso-osmotic solute" is meant a non-ionizing compound dissolved in an aqueous solution.

By "solution that is substantially free of electrolytes" is meant a solution to which no electrolytes have been added or in which the concentration of electrolytes is less than 0.01 or 0.001 mM.

The term "nanoscale" generally refers to structures having dimensions that may most conveniently be expressed in terms of nanometers. For example, the term "nanoscale structure" may refer to a structure having a largest dimension of approximately 500 nm or less, approximately 100 nm or less, approximately 50 nm or less, approximately 20-50 nm, approximately 10-20 nm, approximately 5-10 nm, approximately 1-5 nm, approximately 1 nm, or between 0.1 and 1 nm. "Approximately" as used herein means that the measurement may deviate by 10% from the numeral given, and the ranges listed are assumed to include both endpoints. The relevant dimension may be, e.g., length, width, depth, breadth, height, radius, diameter, circumference, or an approximation of any of the foregoing in the case of structures that do not have a regular two or three-dimensional shape such as a sphere, cylinder, cube, etc. Any other relevant dimension may also be used to determine whether a structure is a nanoscale structure, depending on the shape of the structure. One of ordinary skill in the art will recognize that one or more dimensions of a nanoscale structure need not be in the nanometer range. For example, the length of such structures may run into the micron range or longer.

As used herein, the term "nanofiber" refers to a fiber having a diameter of nanoscale dimensions. Typically a nanoscale fiber has a diameter of 500 nm or less. According to certain embodiments of the invention a nanofiber has a diameter of less than 100 nm. According to certain other embodiments of the invention a nanofiber has a diameter of less than 50 nm. According to certain other embodiments of the invention a nanofiber has a diameter of less than 20 nm.

According to certain other embodiments of the invention a nanofiber has a diameter of between 10 and 20 nm. According to certain other embodiments of the invention a nanofiber has a diameter of between 5 and 10 nm. According to certain other embodiments of the invention a nanofiber has a diameter of less than 5 nm.

The term "nanoscale environment scaffold" refers to a scaffold comprising nanofibers. According to certain embodiments of the invention at least 50% of the fibers comprising the scaffold are nanofibers. According to certain embodiments of the invention at least 50% of the fibers comprising the scaffold are nanofibers. According to certain embodiments of the invention at least 75% of the fibers comprising the scaffold are nanofibers. According to certain embodiments of the invention at least 90% of the fibers comprising the scaffold are nanofibers. According to certain embodiments of the invention at least 95% of the fibers comprising the scaffold are nanofibers. According to certain embodiments of the invention at least 99% of the fibers comprising the scaffold are nanofibers. Of course the scaffold may also comprise non-fiber constituents, e.g., water, ions, growth and/or differentiation-inducing agents such as growth factors, therapeutic agents, or other compounds.

The term "microscale" generally refers to structures having dimensions that may most conveniently be expressed in terms of micrometers. For example, the term "microscale structure" may refer to a structure having a largest dimension of approximately 500 μm or less, approximately 100 μm or less, approximately 50 μm or less, approximately 20-50 μm, approximately 10-20 μm, approximately 5-10 μm, approximately 1-5 μm, approximately 1 μm, or between 0.1 and 1 μm. The relevant dimension may be, e.g., length, width, depth, breadth, height, radius, diameter, circumference, or an approximation of any of the foregoing in the case of structures that do not have a regular two or three-dimensional shape such as a sphere, cylinder, cube, etc. Any other relevant dimension may also be used to determine whether a structure is a microscale structure, depending on the shape of the structure. One of ordinary skill in the art will recognize that one or more dimensions of a microscale structure need not be in the nanometer range.

As used herein, the term "microfiber" refers to a fiber having a diameter of microoscale dimensions. Typically a microscale fiber has a diameter of 500 μm or

less, a diameter of less than 100 μm, a diameter of less than 50 μm, a diameter of less than 20 μm, a diameter of between 10 and 20 μm, or a diameter of between 5 and 10 μm.

The present invention provides a number of advantages related to the repair or replacement of tissues. For example, these methods enable the encapsulation of living cells by a peptide scaffold in a three-dimensional arrangement and in a substantially uniform distribution, which may promote the viability and proliferation of the cells. The cells are present in an architecture that more closely approximates the natural situation of cells in the body than does culture in a traditional plastic culture dish or other two-dimensional substrate. As demonstrated by electron microscopy, the peptide scaffolds comprise a network of nanofibers with intervening spaces rather than a solid matrix. Such a structure may allow cell penetration and cell-cell interaction in a way that more closely resembles the setting of cells within the body than allowed by other culture techniques and materials. The ability of cells to adhere to a substrate may influence cell morphology. (See, e.g., Powers, M. J., Rodriguez, R. E., Griffith, L. G., Cell-substratum adhesion strength as a determinant of hepatocyte aggregate morphology. *Biotech. and Bioeng.* 53, 415-426, 1997). The peptide scaffolds also have the advantage of not eliciting a detectable immune or inflammatory response in mammals. Further, the peptide scaffolds exhibited no detectable swelling when scaffold added to a saline solution. This lack of swelling is probably due to the high water content of the scaffold (typically >99%). This property of the scaffold reduces the probability of an unregulated expansion of the scaffold that could lead to adverse physiological effects on neighboring tissues. Moreover, if desired, the *in vivo* rate of degradation of the scaffolds may be modulated by the incorporation of protease cleavage sites into the scaffold.

III. Cells For Use in the Invention

Any type of cell, progenitor cell, stem cell, etc., is appropriate for use in the present invention. Sources of the cells may also include fetal or adult mammals or established cell lines. Numerous established cell lines are known in the art, many of which are available through the American Type Culture Collection (http://www.atcc.org), which also provides references describing these cell lines. In

discussing cells, progenitor cells, stem cells, and cell lines, the phrase "derived from" indicates that a cell is obtained from a particular source, or that the cell is a descendant of a cell obtained from that source. For example, a liver-derived cell is a cell that is obtained from the liver or the progeny or descendant of such a cell. When the term "progeny" is used herein, it refers not only to the immediate products of cell division but also to the products of subsequent cell divisions, i.e., to cells that are descendants of a particular cell. A cell that is derived from a cell line is a member of that cell line or is the progeny or descendant of a cell that is a member of that cell line. A cell derived from an organ, tissue, individual, cell line, etc., may be modified *in vitro* after it is obtained. Such a cell is still considered to be derived from the original source.

Although the Examples describe isolation and encapsulation of liver progenitor cells and their differentiation or transdifferentiation along liver cell lineage, neural cell lineage and pancreatic cell lineage pathways, the invention is not limited to those embodiments. Stem cells and progenitor cells are known to exist in a wide variety of tissues (See references cited above). Cells may be obtained from any body tissue, organ, or structure. Thus cells may be derived from brain, nervous tissue, bone marrow, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, bladder, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, and uterus. Embryonic stem cells may be used.

As discussed above, mature hepatocytes and other mature liver cell types are able to divide and give rise to daughter cells of the same type. However, the existence, identity, and origin of liver stem cells or liver progenitor cells and their role in liver regeneration remains unclear (See, e.g., Peterson, B. *et al.*, "Bone Marrow as a Potential Source of Hepatic Oval Cells", *Science*, 284, May 14, 1999; Paku, S., *et al.*, "Origin and Structural Evolution of the Early Proliferating Oval Cells in Rat Liver", Am. J. Path., 158(4), 2001.) As described in Example 1, liver progenitor cells may be conveniently harvested from rat liver. In the case of a human, liver cells may be harvested surgically or using a less invasive liver biopsy technique. Liver progenitor cells may be isolated from a mixed population of cells residing in the liver. The bone marrow is likely to be a good source of progenitor

cells having broad transdifferentiation potential (i.e., potential to differentiate into multiple different cell types). Thus the invention specifically contemplates the use of progenitor cells derived from bone marrow.

Cells harvested from an individual may be used either with or without a period of expansion in culture. Alternately, cells that have been propagated in culture as a stable cell line may be used. In certain embodiments of the invention the cells are autologous or allogeneic. In certain embodiments of the invention cells are harvested from a subject, e.g., a patient, and a clonal cell line is derived from one or more of these cells. Clonal lines of progenitor cells, including somatic tissue stem cells may be obtained by limiting dilution plating or single cell sorting. Methods for deriving clonal cell lines are well known in the art and are described for example in Puck, T. T. and Marcus, P. I., J. (1956) Experimental Medicine 103, 653; Nias, A. H. W. and Lajtha, L. G. (1965)" Clone size distribution in the study of inhomogeneity of growth rates in tissue culture" in Cell Culture, C. V. Ramakrishnan, ed. (Dr. W. Junk Publishers, Netherlands), and Leong, P.-M., Thilly, W. G., and Morgenthaler, S. (1985) Variance estimation in single-cell mutation assays: comparison to experimental observations in human lymphoblasts at 4 gene loci. Cells from the cell line are used in the practice of the invention. When intended for treatment of a particular patient, cells from a matched donor may be advantageously used. Cells isolated from an individual or maintained as a cell line may be cultured according to any appropriate technique including standard cell culture techniques prior to their use in the practice of the present invention.

It may be desirable to genetically modify the cells prior to their use in the invention. Numerous methods for introducing exogenous genetic material into cells are well known in the art. In certain embodiments of the invention it may be desirable to introduce a selectable marker into the cells. In certain embodiments of the invention it may be desirable to introduce a gene that encodes a selectable marker (e.g., a gene encoding a protein that confers drug resistance) or a detectable marker (e.g., GFP) under the control of a tissue-specific promoter. Expression of the detectable marker may then be used as a means to determine whether the cell or its progeny has differentiated or transdifferentiated along a particular cell lineage pathway characteristic of that tissue. The marker may also be used as a means of

isolating cells that have differentiated or transdifferentiated along a particular pathway, e.g., by using immunological methods, FACS, etc., such other methods as are well known in the art. Numerous selectable and detectable markers are known in the art. In addition, tissue-specific, organ-specific, and lineage-specific promoters are well known. Genes may be introduced under the control of either a constitutive or an inducible promoter of which many are known in the art.

In certain embodiments of the invention a therapeutically desirable genetic modification may be made. For example, in a case where an individual harbors a mutation in a particular gene it may be desirable to introduce a wild-type copy of the gene into the progenitor cell for gene therapy purposes. This approach may be particularly useful in the case of certain liver diseases (See, e.g., Grompe, M., "Liver repopulation for the treatment of metabolic diseases", *J. Inherit. Metab. Dis.*, 24, 231-244, 2001 for a discussion of some of these diseases.) In certain embodiments of the invention it may be desired to introduce a gene encoding a particular receptor, e.g., a growth factor receptor, in order to confer or enhance a particular differentiation or transdifferentiation potential by allowing cells to respond to the growth factor.

In certain embodiments of the invention it is desirable to enrich for progenitor cells. Various methods of enrichment may be used. For example, the presence of particular markers may be used to remove or otherwise exclude cells that have differentiated and reached a point at which they do not qualify as progenitor cells. Techniques for removing cells or sorting cells are well known in the art and include the use of flow cytometry (e.g., FACS) and various other methods employing antibodies that recognize particular cell types.

In certain embodiments of the invention it may be desirable to employ cells in which asymmetric cell kinetics have been suppressed, as described in detail in the U.S. provisional patent application entitled "Methods for Ex Vivo Propagation of Somatic Stem Cells", filed July 10, 2001, on which one of the present inventors (Dr. James Sherley) is a co-inventor. Suppression of asymmetric cell kinetics (SACK) allows the expansion of a stem cell population without the dilution that inevitably occurs under conditions in which a stem cell gives rise to both another stem cell and a more fully differentiated cell. The technique may be applied to cells harvested

from an individual or to a cell line. A clonal cell line may be established from cells to which SACK has been applied.

IV. Differentiation and Transdifferentiation enhancing Factors and Agents For Use in the Invention

As is well known in the art, numerous environmental factors are likely to play key roles in cell differentiation and transdifferentiation. These may include physical or mechanical factors such as compressive forces, contact with substrate, etc. The extracellular matrix is known to exert profound effects on cell development. In addition, cell-cell contacts may play an important role. In addition, a large number of specific growth and/or differentiation factors have been described. Among these are epidermal growth factor (EGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-I (IGF-I), transforming growth factor β (TGF- β), etc. The foregoing list is merely representative. Over 2,000 growth factors have been identified, and one of ordinary skill in the art will be able to select and to test these factors appropriately depending upon the differentiation or transdifferentiation properties desired. The growth factors may be provided in a pure form or as components of a more complex biological mixture such as serum. The growth factors may be present within the culture medium of a peptide hydrogel structure in which cells are encapsulated and/or may be encapsulated within the structure itself. In addition, it is well known in the art that different concentrations of a particular growth factor may exert different effects on target cells. One of ordinary skill in the art will be able to test a range of concentrations and combinations in order to achieve the desired effects.

Various ECM components may be included in the peptide hydrogel. These include proteins such as fibronectin, laminin, collagens, elastin, etc., and also glycosaminoglycans, e.g., hyaluronic acid, chondroitin sulfate, and dermatan sulfate. Cells cultured within peptide hydrogels in the presence of these ECM components may also be cultured in the presence of these components after removal from the hydrogel.

In addition to growth factors and ECM components, various other chemical stimuli or conditions may influence cell differentiation or transdifferentiation, and any of these may be used in the context of the present invention. Among such stimuli are activators of the phosphatidyl inositol pathway, or other factors that increase levels of inositol trisphosphate and/or intracellular Ca concentrations, activation of protein kinase C and/or other cellular kinases, etc. The presence of small molecules including small organic molecules or metal ions may also influence cell differentiation or transdifferentiation and may be used in the practice of the invention.

V. Monitoring Cell Division and Phenotype

As is well known in the art, there are a number methods for monitoring cell division and for assessing various aspects of cell behavior and phenotype. In general, any appropriate method may be employed to investigate and assess the effects of culturing cells in or on the peptide structures described herein. In addition, the effects of the cells on the overall composition and properties of the cell/hydrogel assembly may be monitored. Such features as protein content, strength, etc., can be examined.

Cell viability may be assessed by examining vital dye exclusion (e.g., trypan blue exclusion). Cell division may be observed by light microscopy and is indicated by the presence of mitotic figures. mRNA and/or protein synthesis may also be measured by techniques well known in the art. An increase in cell number accompanying division may also be observed, e.g., by counting with a hemacytometer. Morphological changes such as cell rounding may also accompany division. DNA synthesis may be monitored by detecting and/or measuring incorporation of various substances such as radiolabeled nucleotides (e.g., ^{3}[H] thymidine), bromodeoxyuridine (BrdU), etc., into DNA.

Cell differentiation and transdifferentiation can be assessed based on a number of parameters, including morphology. Cell differentiation and transdifferentiation may also be assessed by monitoring gene expression (e.g., by detecting and/or measuring mRNA by Northern blot analysis, RT-PCR, microarray analysis, etc.) or by detecting and/or measuring the presence of certain polypeptides

(e.g., using Western blots, immunoprecipitation, ELISA assays, and various functional assays for polypeptide activity). Genes and their expression products (RNA and protein) characteristic of particular cell types or stages, etc., are commonly known as markers. Markers characteristic of numerous different cell types and differentiations states have been identified. In some cases such markers identify a cell as belonging to one of a restricted number of cell types but do not uniquely identify the exact cell type. In other cases the presence of a marker is believed to identify a cell as being of a particular cell type and no other. In addition to allowing identification of cell type, certain markers are characteristic of cells that lack or possess a particular feature or functional capability (e.g., cells that are post-mitotic).

The variety of markers is immense, and new markers are routinely being identified. Of particular significance in the context of the present invention are markers that may be used to identify cells that are able to differentiate or transdifferentiating into cell types characteristic of the mature liver and markers that may be used to identify cells that have differentiated into such cells. Also of particular significance in the context of the present invention are markers that may be used to identify cells capable of differentiating or transdifferentiating along a neuronal lineage pathway, i.e., a pathway that ultimately results in the production of neurons or glia. Also of particular significance in the context of the present invention are markers that may be used to identify cells capable of differentiating or transdifferentiating along a pancreatic lineage pathway, e.g., a pancreatic endocrine or exocrine cell pathway including, but not limited to, an insulin-secreting beta-cell like pathway .

A variety of markers for liver precursor cells and for cells that are found in the mature liver are known in the art and are described, for example, in Grisham, J. and Thorgeirsson, S., "Liver stem cells", in Potten, C. (ed.), *Stem Cells*, Academic Press, San Diego, 1997. A selection of markers appropriate for assessing differentiation along a liver cell pathway include the embryonic liver and oval cell marker alpha-fetoprotein (Shiojiri, N., Lemire, J.M. & Fausto, N. "Cell lineages and oval cell progenitors in rat liver development", *Cancer Res.* **51**, 2611-2620, 1991); the hepatocyte and oval cell marker albumin (Houssaint, E. "Differentiation of the

mouse hepatic primordium. I. An analysis of tissue interactions in hepatocyte differentiation". *Cell Differ.* 9, 269-279, 1980); various cytochrome P450 enzymes including CYP1A1 and CYP1A2 (present in hepatocytes, but nonspecific); the liver developmental marker C/EBPα; a CCAAT enhanced-binding protein highly expressed in hepatocytes and other endodermal tissues (Wang, N.D., Finegold, M.J., Bradley, A., Ou, C.N., Abdelsayed, S.V., Wilde, M.D., Taylor, L.R., Wilson, D.R. & Darlington, G.J. "Impaired energy homeostasis in C/EBP alpha knockout mice", *Science* 269, 1108-1112, 1995); a marker for all hepatic cells (hepatocytes, biliary duct and oval cells) cytokeratin 18 (CK18) (Van Eyken, P., Sciot, R. & Desmet, V. "Intrahepatic bile duct development in the rat: A cytokeratin-immunohistochemical study", *Lab. Invest.* 59, 52-59, 1988); and specific biliary duct markers cytokeratin 19 (CK19) (Bouwens, L., Wang, R.N., De Blay, E., Pipeleers, D.G., & Kloppel, G. "Cytokeratines as markers of ductal cell differentiation and islet neogenesis in the neonetal rat pancreas", *Diabetes* 43, 1279-1283, 1994) and cytokeratin 7 (CK7) (Shiojiri, et al., Grompe, et al.); and the hepatocyte marker cytokeratin 8 (CK8) (Grompe, et al., Van Eyken, et al., Bouwens, et al). The presence of binucleated cells is indicative of hepatocytes. A variety of markers for neuronal lineage cells are mentioned in Woodbury, D., *et al.*, J. Neurosci. Res. 61: 364-370, 2000 and in Mahendra S. Rao (ed.) *Stem cells and CNS development*, Totowa, N.J. : Humana Press, 2001.

Among the markers appropriate for assessing differentiation or transdifferentiation along a neuronal lineage pathway are nestin, GFAP, and NeuN. Nestin is an intermediate filament protein expressed in neuroepithelial neuronal precursor stem cells, and its expression decreases with neuronal maturation (Lendahl, U., *et al.*, "CNS stem cells express a new class of intermediate filament protein", *Cell*, 60:585-595, 1990. NeuN is a neuron-specific marker expressed in postmitotic cells (Sarnat, H., *et al.*, "Neuronal nuclear antigen (NeuN): a marker of neuronal maturation in early human feta nervous system", *Brain Research*, 20:88-94, 1998). Glial fibrillarary acidic protein (GFAP) is a classic glial astrocyte marker. These markers have been employed to demonstrate the applicability of certain embodiments of the invention (see Examples). Numerous other markers for neuronal lineage cells are known in the art.

Among the markers appropriate for assessing differentiation or transdifferentiation along a pancreatic lineage pathway are the various polypeptide hormones produced by pancreatic endocrine cells, including insulin I, insulin II, glucagon, somatostatin, and pancreatic polypeptide. Additional markers associated with endocrine pancreas development include, but are not limited to, glut 2, PDX-1, Nkx2.2, Nkx6.1, PAX-4, and PAX-6 (Yang, et al., and references therein).

VI. Methods of Treatment

The invention provides a number of methods of treating subjects suffering from various conditions or disorders. In particular, the invention provides a method of treating an individual comprising (i) identifying an individual in need of treatment; and (ii) administering a nanoscale environment scaffold or structure encapsulating cells to the individual. The nanoscale environment scaffold encapsulating cells may be any of the nanoscale environment scaffolds or structures described herein. In particular, the nanoscale environment scaffold encapsulating cells may comprise or consist of stem cells or progenitor cells. According to certain embodiments of the invention the cells comprise liver cell lineage cells, neural lineage cells, or pancreatic lineage cells. According to certain embodiments of the invention the cells comprise stem cells or progenitor cells that have been instructed or induced to differentiate. The cells may be instructed or induced to differentiate along a liver cell lineage pathway, along a neural lineage pathway, and/or along a pancreatic lineage pathway. The cells may comprise liver cells (e.g., liver stem cells, liver progenitor cells, hepatocytes, oval cells, bile duct cells), neural lineage cells (e.g., neurons or glia), and/or pancreatic lineage cells (e.g., endocrine cells such as β, α, γ, or δ cells or exocrine cells). The structure encapsulating cells may contain one or more differentiation enhancing agents such as ECM components and/or growth factors.

Another method comprises steps of (i) culturing progenitor cells or stem cells in a peptide hydrogel thereby producing a peptide hydrogel structure containing cells; and (ii) implanting the peptide hydrogel structure containing cells into a subject. The peptide hydrogel may contain one or more differentiation enhancing agents such as a growth factor or ECM component. Alternately, such an agent may

be present in the medium in which the cells are cultured instead of or in addition to being present within the hydrogel. The method may further include a step of allowing the cells to differentiate or transdifferentiate while within the peptide hydrogel. In particular, according to certain embodiments of the invention the cells differentiate along a hepatocyte lineage pathway, a neural cell lineage pathway, or a pancreatic cell lineage pathway such as an insulin secreting β-like cell pathway. Cells that have differentiated along a hepatocyte lineage pathway and structures containing such cells are particularly useful for treatment of disorders and diseases of the liver including, but not limited to, physical or chemical injury, hepatitis, liver cancer, and cirrhosis. Cells that have differentiated along a neural cell lineage pathway and structures containing such cells are particularly useful in treating disorders and conditions of the central or peripheral nervous system including injury, degenerative diseases, tumors, Parkinson's disease, multiple sclerosis, Alzheimer's disease, epilepsy, stroke, etc. Cells that have differentiated along a pancreatic cell lineage pathway and structures containing such cells are particularly useful for treating disorders or diseases of the endocrine and/or exocrine pancreas including, but not limited to, diabetes, pancreatitis, pancreatic cancer, and cystic fibrosis. Cells that are capable of secreting insulin are useful in particular for treating conditions characterized by a relative or absolute lack of insulin functional activity or characterized by insulin resistance, including, but not limited to, type I or type II diabetes.

According to certain embodiments of the invention at least a portion of the cells cultured in the hydrogel in the presence of particular differentiation enhancing agents produce insulin at a greater level than comparable cells cultured in the hydrogel in the absence of the differentiation enhancing agent(s). According to certain embodiments of the invention at least a portion of the cells cultured in the hydrogel in the presence of particular differentiation enhancing agents produce insulin at a level at least 5% as much insulin per milligram protein as that produced by normal islet cells. According to certain embodiments of the invention at least a portion of the cells cultured in the hydrogel in the presence of particular differentiation enhancing agents produce insulin at a level at least 10% as much insulin per milligram protein as that produced by normal islet cells. According to

certain embodiments of the invention at least a portion of the cells cultured in the hydrogel in the presence of particular differentiation enhancing agents produce insulin at a level at least 20% as much insulin per milligram protein as that produced by normal islet cells. According to certain embodiments of the invention at least a portion of the cells cultured in the hydrogel in the presence of particular differentiation enhancing agents produce insulin at a level at least 50%, at least 75%, or as much as 100% as much insulin per milligram protein as that produced by normal islet cells. According to certain embodiments of the invention at least a portion of the cells cultured in the hydrogel in the presence of particular differentiation enhancing agents secrete insulin in response to various stimuli including glucose, IBMX, carbachol, and/or tolbutamide.

According to another method, cells are cultured within a peptide hydrogel structure under any of a variety of conditions, e.g., in the presence of one or more differentiation enhancing agents such as a growth factor or an ECM component and are then removed from the hydrogel. Cells are then implanted into a subject in need of treatment, either immediately or after a period of culture, e.g., in a conventional culture vessel.

The peptide structures encapsulating cells, or cells isolated from the peptide structures after a period of culture therein may be used in the construction of artificial organs, e.g., artificial liver or pancreas

VII. Additional Embodiments

The invention provides kits that may be used for enhancing cell differentiation and/or transdifferentiation. The kits comprise a peptide hydrogel of the invention, which may be provided in dry or lyophylized form. The kits may further comprise one or more of the following elements: instructions for encapsulating cells within a peptide hydrogel structure and for other uses of the system, instructions for inducing cells to differentiate or transdifferentiate within the scaffold, a vessel in which the encapsulation may be performed, a liquid in which the peptide can be dissolved, an electrolyte for initiating peptide self-assembly, medium for tissue culture, cells that may be encapsulated, differentiation-enhancing agents, etc. Additional elements may also be included.

In addition, the invention provides assay systems and methods for testing compounds. Since the liver is the major organ that metabolizes a wide range of foreign and endogenous compounds including drugs, it is great importance to determine the effects of such compounds on the liver, e.g., their ability to induce liver enzymes, and the ability of the liver to metabolize such drugs. Traditionally such assessments have initially been performed using *in vitro* systems such as microsomes, or *in vivo* animal models. However, these approaches have significant drawbacks. The microsome system does not allow for tests that include the possible effects of cell membrane barriers or other features of an intact cell. Animal models are expensive and time-consuming to employ. In addition, animal liver cells may metabolize compounds differently to human liver cells due, for example, to a different cytochrome P450 profile. The difficulties of culturing hepatocytes *in vitro* have precluded the development of cell-based assay systems.

The present invention provides an assay system for a compound comprising a peptide hydrogel structure encapsulating liver progenitor cells (non-human animal or human), wherein the liver progenitor cells have been induced to differentiate along a hepatocyte pathway and exhibit features of a mature hepatocyte such as expression of metabolic enzymes such as cytochrome P450 enzymes. According to the invention a compound is applied to the system, and various parameters are tested. For example, the ability of the encapsulated cells to metabolize the compound may be tested. The ability of the compound to induce or inhibit P450 enzymes may be assessed, e.g., using a fluorescent or otherwise conveniently detectable substrate such as that as described in the Examples. The effect of the compound on other liver parameters, e.g., the synthesis of proteins such as albumin, alanine transaminase, aspartate transaminase, etc., may be assessed. Methods for measuring a wide variety of proteins synthesized by the normal liver are well known in the art. Many drugs and other foreign compounds are known to induce or inhibit liver enzymes, and such effects present significant safety concerns in terms of drug development. It is desirable to test the effects of new medication candidates upon liver enzymes. The present invention provides a way of performing such tests *in vitro*, which may be used to predict potential dangers posed by new drug candidates, drug interactions, etc.

VIII. Equivalents

The representative examples which follow are intended to help illustrate the invention, and are not intended to, nor should they be construed to, limit the scope of the invention. Indeed, various modifications of the invention and many further embodiments thereof, in addition to those shown and described herein, will become apparent to those skilled in the art from the full contents of this document, including the examples which follow and the references to the scientific and patent literature cited herein. The following examples contain important additional information, exemplification and guidance which can be adapted to the practice of this invention in its various embodiments and the equivalents thereof.

EXAMPLES

Example 1

Encapsulation of Adult Rat Liver Precursor Cells in Peptide Hydrogels

Materials and Methods

Rat somatic liver progenitor/stem cells. The isolation and preparation of the liver progenitor cells is described in detail in U.S. provisional patent application "Methods for Ex Vivo Propagation of Somatic Stem Cells", filed July 10, 2001, on which one of the present inventors (Dr. James Sherley) is a co-inventor. Briefly, xanthosine (Xs) was used as a pharmacological agent allowing for a switch from the default asymmetric kinetics normally exhibited by tissue stem cells to exponential kinetics. When xanthosine was removed, clonal rat liver epithelial stem cell lines that retained the ability to divide by asymmetric cell kinetics were isolated. Rat liver epithelial cells found in the low-speed supernatant of centrifuged cells from *in situ* collagenase-perfused livers were isolated by limiting dilution cloning in the absence or presence of Xs. The cell line used in this work, which is termed LPC-8, LPC-8.1 (a sub-clone of LPC-8), or Xs-D8, or D8 elsewhere herein and in the above-mentioned provisional patent application, has now been maintained continuously in culture for more than 80 cell doublings. Early passage cells were cryo-preserved in liquid nitrogen and can be reestablished in culture after thawing.

Encapsulation of LPC-8 Cells. Cells were maintained in plastic culture dishes with DMEM supplemented with 10% dialyzed fetal bovine serum (FBS), penicillin, and streptomycin. When cells reached 80% confluence they were harvested by treatment with trypsin. The cell suspension was washed with DMEM/10%FBS/pen/strep and then resuspended in an aqueous solution of 10% sucrose. Cells were counted using a hemacytometer.

RAD16-I peptide (sequence AcN-RADARADARADARADA-CNH_2) [theoretical MW=1712.74 and MW by Mass spectra=1712.64] was obtained from ResGen Invitrogen Corporation. A RAD16-I peptide solution was prepared by dissolving peptide in deionized, distilled, sterile water at a concentration of 0.5% w/v. The RAD16-I solution was equilibrated with PBS (phosphate buffered saline) to a final concentration of 1X prior to mixing with the cell suspension in order to bring the pH within a physiologic range.

Cells were mixed with RAD16-I solution at a final concentration of approximately 100,000 cells/ml. The cell/peptide mixture was loaded into multiwell (96-well) plates at 50 µl/well. Immediately after loading, 200 µl of culture medium (DMEM/10%FBS/pen/strep) was added to each well, thereby providing an electrolyte concentration sufficient to allow self-assembly of the gel into a three-dimensional structure. After gel self-assembly, the media was changed three to four times to allow proper equilibration of the cell/hydrogel assembly. The final cell density was between 1-2 x 10^5 cells/ml of hydrogel. The multiwell plates were cultured at 37° C in a standard incubator containing a humidified chamber equilibrated with 5% CO_2. Cell viability was measured by staining with trypan blue according to standard techniques. Viability 24 hours after encapsulation was typically greater than 85%.

Control Cells. Control cultures of LPC-8.1 cells were initiated at the same time as the encapsulation using a portion of the cell/sucrose suspension. Cells were maintained on plastic culture dishes in DMEM/10%FBS/pen/strep at 37° C in a standard incubator containing a humidified chamber equilibrated with 5% CO_2. Cell

viability was measured by staining with trypan blue according to standard techniques. Viability 24 hours after plating was typically greater than 90%.

LPC-8 cells were also grown on the surface of plates coated with assembled RAD16-I hydrogel in DMEM/10%FBS/pen/strep and maintained at 37°C in 5% CO_2.

LPC-8 cultures in soft agar were also prepared according to standard protocols.

BrdU Staining. Cell division within the assembled peptide hydrogel was assessed by monitoring incorporation of 5'-bromodeoxyuridine (BrdU). BrdU was added to the culture medium at a final concentration of 10 μM for a period of 18 hours. Following incubation, hydrogel cultures were incubated in BrdU-free medium for two hours. Hydrogels were then washed in PBS, followed by fixation in 2% paraformaldehyde in PBS (pH 7.4) at room temperature for 2 hours. Following fixation, hydrogels were washed several times in PBS and then treated with 0.1% Triton X-100 in PBS for 2 hours at room temperature. To achieve DNA denaturation, hydrogels were then treated with 2N HCl in PBS for 30 minutes at 37° C . Following this treatment, several washes with PBS were performed until a pH of 7.4 in the wash solution was reached. Hydrogels were then incubated in blocking buffer (20% FBS, 0.1% Triton X-100, 1% DMSO in PBS) for four hours at room temperature with slow shaking.

FITC-conjugated, anti-BrdU mouse monoclonal antibody IgG_1 (BD Pharmingen, catalog number 33284X) was preincubated in blocking buffer for one hour at room temperature at a dilution of 1:400 and then added to samples overnight at 4° C with slow shaking. Following incubation, hydrogels were washed three times with blocking buffer for two hours, following which a final one hour wash with PBS was performed.

Light Microscopy and Photography. Control cells and hydrogels were observed under a Nikon microscope TE300 with phase contrast and fluorescence using an Openlab acquisition system mounted with a Hamamatsu video camera. Pictures obtained represent a single optical plane observed with phase contrast and fluorescence.

Results

Encapsulated and control cells were observed daily by light microscopy. The transparency of the assembled peptide structure readily allowed observation and photography of encapsulated cells. Immediately after encapsulation, the cells were substantially uniformly dispersed throughout the gel as isolated single cells rather than in groups or clumps. Figure 3a shows encapsulated LPCs immediately after encapsulation, illustrating uniform, single-cell dispersion. When cells divide symmetrically in three dimensions, they tend to form compact, spheroidal clusters. However, if cell division is asymmetric, since only one cell of the initial cluster divides, a linear or sometimes branched cluster is formed. Figure 3b shows encapsulated LPCs two days after encapsulation. As shown in Figure 3b, during the first two days of culture encapsulated cells started forming small linear clusters suggesting some asymmetric mitotic activity. Over the next two to three days the clusters enlarged and adopted a spherical shape. Figure 3c shows spheroid formation four to five days after encapsulation.

Cells cultured on plates coated with RAD16-I peptide hydrogel behaved similarly to those cultured directly on the plastic plate surface, continuing to divide and showing no evidence of differentiation or cluster formation. No formation of clusters or other evidence of cell division was observed in the soft agar cultures, indicating that a three-dimensional environment alone is not permissive for cell division.

Incorporation of BrdU indicated that a fraction of the cells in clusters that developed in the peptide structures were actively dividing. Figure 3d shows the same spheroid as in Figure 3c, after staining for incorporation of BrdU into DNA. Clusters typically reached an average cell number of approximately 20-30, at which time further increase in cluster size was not observed. However, the possibility that one or two cell within the cluster continued to divide at a low rate cannot be excluded and is, in fact, suggested by the facts that (as described below), not all cells in the cluster are positive for CYP1A1, and when clusters are extracted from hydrogels and plated on flat dishes where the morphological changes that accompany cell division are readily observed, one or two cells continue to divide.

Example 2

Differentiation Properties of Encapsulated Cells

Materials and Methods

Cell Culture. Cells were cultured in standard plastic culture dishes and encapsulated in peptide hydrogels as described in Example 1. Controls and encapsulated cells were initially maintained in identical media (DMEM/10%FBS/pen/strep) and under identical culture conditions. For some experiments cells that had been cultured in DMEM/10%FBS/pen/strep for one week were switched to a defined hepatocyte growth medium (HGM: Base medium: DMEM from Gibco, #11054-020 (500ml), 0.015 g L-Proline, Sigma # P-4655, 0.05 g L-Ornithine, Sigma # O-6503, 0.305 g Niacinimide, Sigma # N-0636, 0.5 g D-(+)-Glucose, Sigma # G-7021, 1 g D-(+)-galactose, Sigma # G-5388, 1 g Bovine Serum Albumin, fraction V, Sigma # A-9647; 500 ml of trace metal solutions: ZnCl2: 0.0272 g, ZnSO4-7 H2O: 0.0375 g, CuSO4-5 H2O: 0.01 g, MnSO4: 0.00125 g; 12.5 ml L-Glutamine, final concentration [5 mM], Gibco # 25030-081, 0.5 ml Insulin-Transferin-Sodium Selenite, Roche # 1074547, 0.4 ml Dexamethasone, final concentration [0.1 microM], Sigma # D-8893, 5 ml penicillin/streptomycin (100 x solution), Epidermal Growth Factor (EGF) final concentration [20 ng/ml], Collaborative # 40001). For other experiments, cells that had been cultured in DMEM/10%FBS/pen/strep for one week were switched to DMEM/pen/strep containing 1% FBS. For some experiments LPC-8.1 cells were induced to form floating spheroids by resuspending trypsinized cells in glass spinner flasks at a density of 10^6 cells per 30 mL.

Assessment of Cytochrome P450 1A1 activity. After two weeks, cultures were incubated in the presence of 7-ethoxyresofurin, a specific substrate for the enzyme whose cleavage produces the fluorescent residue resofurin (excitation: 574 nm, emission: 596 nm). The incubations were carried out at 37 °C for 30 minutes. After incubation the hydrogels were washed three times with culture media. Cells were then monitored under a Nikon microscope TE 300 with phase contrast and fluorescence using an Openlab data acquisition system mounted with a Hamamatsu video camera. Pictures obtained represent a single optical plane observed with

phase contrast and fluorescence. For all experiments multiple clusters were observed, and results represent typical observations.

For quantitative measurement of CYP1A1 activity, 7-ethoxyresofurin O-deethylase activity (EROD) was measured in culture supernatants as previously described (Tokudome, S., Yamamoto, T., and Kuroiwa, Y. Involvement of CYP1A2 in mexiletine metabolism. Br. J Clin Pharmacol 46, 55-62, 1998). Briefly, the two types of culture were incubated in the presence 0.1 mM of 7-ethoxyresofurin at 37°C for 30 minutes, following which the supernatant was harvested. The presence of the product resofurin was measured fluorometrically. Known concentrations of the standard (resofurin) were used to generate a standard curve. The total number of cells present in both culture dishes and in the peptide hydrogels was determined using a hematocytometer. EROD activity was expressed as pM of resofurin/cell/hr. Approximately 100 clusters were analyzed.

Results

In order to determine whether the cells were differentiating into hepatic lineages, Cytochrome P450 1A1 (CYP1A1) enzyme activity, characteristic of fully differentiated hepatocytes, was analyzed over time both visually and using a quantitative assay in cells grown on plastic culture plates and in cells encapsulated within the peptide hydrogel structure. As shown in Figure 4a, LPCs growing as a monolayer on a standard plastic culture dish did not display detectable EROD activity two weeks after plating. In contrast, as shown in Figure 4b, LPCs in spheroids growing over the same time period in an assembled peptide structure exhibited abundant EROD activity at two weeks as evidenced by the red staining seen within cells. All spheroids examined contained EROD positive cells. The percentage of positive cells within a cluster ranged between approximately 50 and 80 percent.

EROD activity in cultures of encapsulated LPC-8.1 cells was undetectable one day after encapsulation but rose dramatically by three days, reaching a maximum of approximately 0.14 pmol/cell/hr by day seven. Activity declined slightly thereafter but remained at approximately 0.08 pmol/cell/hr on day 15. Figure 4c is a graph showing EROD activity of LPC spheroids growing in an

assembled peptide structure during a time course of two weeks, starting 24 hours after encapsulation. The slope of the graph suggests that EROD activity was reaching a plateau by two weeks following encapsulation.

Switching control cells growing on standard tissue culture plates to hepatocyte growth medium had no effect on EROD activity, which remained at essentially undetectable levels after one week of culture in HGM. In contrast, switching encapsulated cells to HGM resulted in a 3 to 4 fold increase in EROD activity after one week of culture relative to the level of EROD activity in encapsulated cells maintained in DMEM/10%FBS/pen/strep. Switching encapsulated cells to HGM also resulted in a dramatic change in cellular morphology in a fraction of the cells, as described in Example 3. EROD activity was also examined in LPC-8.1 cells grown on standard tissue culture dishes under serum starvation conditions (DMEM with 1%FBS) and in LPC-8.1 cells induced to form floating spheroids. EROD activity in serum-starved cells and cells that formed floating spheroids remained at extremely low levels when measured one week after plating. Figure 4d is a graph summarizing the data on comparative CYP1A1 activity of LPC-8.1 cells maintained under these various culture conditions: monolayer on plastic dish with low (1%) serum concentration (serum starvation); spheroid culture obtained by growing LPCs in liquid culture at high density; spheroid culture in assembled peptide structure growing in DMEM with 10% FBS; spheroid culture in assembled peptide structure growing in HGM.

To the inventors' knowledge, the LPC-peptide hydrogel system is the first described *in vitro* model for transdifferentiation by a clonal stem cell that retain differentiation properties after long-term growth in culture.

Example 3

Effects of Growth Factors on Cells Encapsulated in Peptide Hydrogel Structures

Materials and Methods

Cells and Cell Culture. LPC-8.1 cells were grown either in DMEM/10% FBS/pen/strep as above or in hepatocyte growth medium. For some experiments human Epidermal Growth Factor (EGF) at final concentration of 20ng/mL (R&D

Systems, catalog number: 236-EG-200), rat Beta Neural Growth Factor (β-NGF) at final concentration of 5 ng/mL (R&D, Systems, catalog number: 556-NG-100), and human Platelet Derived Growth Factor (PDGF) at final concentration of 10ng/mL (R&D, System, catalog number: 120-HD-001) was added to the medium (either DMEM or HGM).

P19 and NIH3T3 cells were cultured according to standard protocols as previously described by the American Type Culture Collection (ATCC) (see http://www.atcc.org). Retinoic acid treatment of P19 cells to induce neuron/glia differentiation was performed as previously described (Bain, G., *et al.*, "Neuronlike Cells Derived in Culture from P19 Carcinoma Embryonal Stem Cells", in *Culturing Nerve Cells*, 2nd edition, Banker, G. and Goslin, K, (eds.), 1998).

Immunostaining. Immunostaining experiments were performed on hydrogel cultures or in cell cultures on laminin-coated cover slips using the following neuronal markers: Nestin and β-tubulin III for neuronal precursors, NeuN for post-mitotic neurons, and GFAP for glia (astrocytes). The samples (hydrogels or laminin-coated cover slips containing cultured cells) were first fixed in 2% paraformaldehyde in PBS (pH 7.4) for 2 hours at room temperature and subsequently washed several times in PBS. They were then treated with 0.1% Triton X-100 in PBS for two hours at room temperature. Following the treatment, the samples were incubated for a minimum of two hours in blocking buffer with slow shaking (20% Fetal Bovine Serum; 0.1% Triton X-100; 1% DMSO in PBS). The primary antibody in each case was preincubated in blocking buffer for one hour at room temperature (normally at final concentration of 1μg/ml) and then added to the samples and incubated overnight at 4 C with slow shaking. The primary antibodies used were: Goat polyclonal IgG anti-GFAP (Santa Cruz Biotechnology, CA, catalog number: sc-6170); Mouse monoclonal IgG_1 anti-NeuN (CHEMICON International, Inc., catalog number: MAB377); Mouse monoclonal IgG_1 anti-Nestin (CHEMICON International, Inc., catalog number MAB353); and Mouse monoclonal anti-β-tubulin III (CHEMICON International, Inc.). Following the incubation, the samples were washed several times with blocking buffer and subsequently incubated with the appropriate secondary antibodies (1/500 dilution in blocking buffer) overnight at

4 C with slow shaking. The secondary antibodies used were: Goat anti-mouse IgG Rhodamine-conjugated (Santa Cruz Biotechnology); Goat anti-mouse IgG FITC-conjugated (Santa Cruz Biotechnology), and donkey anti-goat –FITC-conjugated (Santa Cruz Biotechnology). The samples were then washed three times with blocking buffer for 2 hours. One final wash with PBS for another hour concluded the treatment. The hydrogels were then monitored under a Nikon microscope TE 300 with phase contrast and fluorescence using an Openlab data acquisition system mounted with a Hamamatsu video camera. Three independent clusters including a total of approximately 100 cells were observed for each staining. Pictures obtained represent one single optical plane observed with phase contrast and fluorescence for FITC and Rhodamine.

BrdU Staining. BrdU staining was performed as in Example 2.

Results

By 24 hours after switching encapsulated LPC-8.1 cells to hepatocyte growth medium, a significant proportion of the cells (10-20%) acquired a dramatic change in cellular morphology, consisting of very elongated cell bodies with rudimentary processes resembling neuronal lineages. Figure 5 shows LPC-8.1 cells growing in an assembled peptide structure after staining for various neuronal markers. The neuronal-like cellular morphology is clearly visible in the phase contrast micrographs in the panels on the left side of the figure (a, c, d, and g). In contrast, cells maintained in culture on standard tissue culture plates exhibited no similar change in morphology when switched to HGM.

To further explore the neuronal-like phenotype, cells growing in peptide hydrogel structures were stained with a variety of antibodies to markers characteristic of neuronal-lineage cells. Figures 5b and 5d show that encapsulated LPC 8.1 cells stained positively for the neuronal progenitor markers Nestin and β-tubulinIII respectively. Most of the cells exhibiting a neuronal morphology stained positive for Nestin and β-tubulinIII. However, encapsulated LPC 8.1 cells stained very poorly for NeuN, a marker for differentiated, post-mitotic neurons (Figure 5f). Encapsulated LPC 8.1 cells were negative for GFAP, a marker for mature,

differentiated glial cells (astrocytes). These results indicated that cells in the hydrogel with neuronal morphology have a phenotype more closely related to early neuronal progenitors than to mature neurons of glia. Cells maintained on standard tissue culture plates in HGM were negative for all four markers.

The morphological analysis and marker staining results suggested that encapsulation within the peptide hydrogel structure provided an environment that allowed the cells to alter their differentiation potential in response to extracellular factors present within HGM. The results suggested that the environment of the hydrogel rendered the cells permissive to instruction by differentiation-inducing factors, e.g., growth factors. Since the defined HGM contains 20 ng/ml of epidermal growth factor (EGF), the possibility that the morphological changes were being induced by EGF was explored by comparing encapsulated cells cultured in HGM with encapsulated cells cultured in either HGM without EGF, DMEM/10%FBS without added EGF (i.e., standard medium) or DMEM/10%FBS with 20 ng/ml EGF added.

When encapsulated cells were cultured in HGM lacking EGF, no change in cellular morphology was observed and most of the cells died within the first 48 hours (see Table 2, which summarizes the results described in Examples 1, 2, and 3, including effects on cell viability, morphology, and staining for neuronal markers when cells were cultured either on plates or in peptide hydrogel structures in either DMEM or HGM with a variety of added factors). This result indicated that the cells were dependent on EGF for survival and suggested that the low concentration of EGF known to be present in FBS is adequate to support survival but not to induce neuronal differentiation when encapsulated cells are cultured in DMEM/10% FBS.

Neither NGF or PDGF was able to support cell viability or induce morphological changes when encapsulated cells were cultured in HGM lacking EGF or in DMEM lacking serum. However, when NGF alone was added to encapsulated hydrogel cultures growing in DMEM with 10% FBS, a similar dramatic change in cell morphology as occurred in HGM medium was observed, i.e., within 24 hours approximately 10-20% of the cells exhibited a neuronal-like appearance with elongated cell bodies with rudimentary processes. Furthermore, when NGF was present in either DMEM/10% FBS, DMEM/EGF, or HGM/EGF it clearly promoted

the number of cells and clusters exhibiting a neuronal morphology, suggesting that the NGF/EGF combination exerts greater neuronal-inducing effects than EGF alone. The presence of PDGF did not induce similar morphological changes either in the presence or absence of EGF. No change in cell morphology was observed when LPC 8.1 cells were cultured in parallel on plastic tissue culture plates in either HGM or DMEM/10% FBS with or without added EGF, NGF, or both.

These results suggested that in the context of the peptide hydrogel structure EGF, while necessary at a low concentration to support cell survival, was able to act as a differentiation-inducing factor at high doses. In contrast, NGF was not able to support cell survival, but in the presence of sufficient EGF to permit cell viability, was able to act as a differentiation-inducing factor. The fact that identical combinations of growth factors and media conditions did not induce either morphological changes or expression of markers for neuronal progenitors when cells were grown on standard culture dishes suggested that the peptide hydrogel structure provided an environment that rendered the cells permissive to instruction by EGF, NGF, or a combination of the two.

In many cases, cells with a neuronal morphology were associated with other cells not exhibiting a neuronal phenotype in small clusters, suggesting the presence of self-renewing neural progenitors. To investigate the possibility of ongoing cell division, cells cultured in HGM in the presence of EGF or EGF plus NGF were independently stained for either Nestin or BrdU. As shown in Figure 6, clusters containing cells with neuronal morphology stained positive for both Nestin (6b, 6f) and BrdU (6d, 6h). Nestin positive staining was associated mainly with cells that exhibited a neuronal phenotype (Figures 6a, b, e, and f) while most BrdU positive cells were not Nestin positive. BrdU positive cells tended to be randomly located within the clusters and exhibited a more spherical morphology. In some cases BrdU staining was observed in cells showing a neuronal morphology. The presence of NGF resulted in an enhancement of the number of cells exhibiting a neuronal phenotype.

Table 2. Peptide hydrogel cultures permit differentiation of somatic liver progenitor cells.

Test	Media	Growth Factor	Viability (%)	Morphology *	Phenotype**
a.	Hydrogel				
1	10% FBS	-	> 85	Spheroid	CYP1A1
2	10% FBS	EGF	> 94	Spheroid/Neural ~60/40	CYP1A1/Nestin/β-tubulin III
3	10% FBS	NGF	> 84	Neural	Nestin/β-tubulin III
4	10% FBS	EGF + NGF	> 95	Spheroid/Neural ~30/70	CYP1A1/Nestin/β-tubulin III
5	HGM	-	< 1	-	
6	HGM	EGF	> 85	Spheroid/Neural ~50/50	CYP1A1/Nestin/β-tubulin III
7	HGM	NGF	< 5	-	-
8	HGM	PDGF	< 5	-	-
9	HGM	EGF + NGF	> 86	Spheroid/Neural ~20/80	CYP1A1/Nestin/β-tubulin III
b.	Culture Dish				
10.	10% FBS	-	>90	Flat/Small	None
11.	10% FBS	EGF	> 95	Flat/Small	None
12.	10% FBS	EGF + NGF	> 95	Flat/Small	None
13	HGM	-	< 1	-	-
14	HGM	EGF	> 90	Flat/Small	None
15	HGM	EGF + NGF	> 90	Flat/Small	None

*By microscopic observation on phase contrast.

**By immunostaining with different antibodies against the specific neuronal markers (Nestin, β-tubulin III) or by analyzing the CYP1A1 activity in vivo with 7-Ethoxyresofurin.

Example 4

Analysis of Cells Extracted from Peptide Hydrogel Structures

Materials and Methods

Cell and spheroid extraction from the hydrogels. In order to isolate cells and spheroids from the hydrogel cultures they were disrupted mechanically with a Pasteur pipette by several up and down aspirations until about 50% of the cells/clusters were extracted as judged by visual inspection under microscope. The extracted mix was placed on Laminin-coated cover slips (Becton & Dickinson) and incubated overnight in the same media in which they had been cultured. The day after extraction, the remaining pieces of hydrogel were removed by washing the cover slips with fresh media and the attached cells/clusters were incubated for a week in an incubator at 37 °C equilibrated with 5% CO_2. Typically about 50% of the cells/clusters were removed from the hydrogels as judged by counting the number of cells remaining in the hydrogel (using a hemacytometer) and counting the attached cells on the cover slips.

Results

To study the behavior and phenotype of the peptide-encapsulated cells in more detail and to explore whether the effects of the hydrogel were reversible, LPC-8 cells that had been growing for one week within a peptide hydrogel structure in HGM containing EFG and NGF were extracted from the hydrogels with mechanical disruption and plated onto laminin-coated plates with the same media and growth factor combinations used with the hydrogel. Cells on the plates acquired two basic morphologies over the course of a week: (1) classical hepatocyte shape with expanded cell bodies and mono- or bi-nuclear appearance (Figure 7a); (2) flat, expanded, with some processes (Figure 7b). At one week after plating, all cells on the plate stained negative for both Nestin and β-tubulinIII. Cells of class 1 (hepatocyte-like morphology) stained negative for both NeuN and GFAP but did exhibit CYP1A1 activity. In contrast, cells of class 2 (glia-like) stained positive for GFAP (Figure 7d, 7f) and negative for NeuN and did not exhibit CYP1A1 activity.

Extensive cell division occurred on the plate, presumably representing division of undifferentiated progenitor cells but not fully differentiated hepatocytes or glia.

These results suggested that the progenitor neuron-like cells derived from LPCs undergo a further differentiation process after extraction from the gels, apparently along a glial pathway. Consistent with the lack of expression of NeuN, no formation of cells with a typical mature neuron morphology (i.e., cells with small cell body and extensive neurites) was observed. The continued expression of CYP1A1 activity and the hepatocyte-like morphology of class 1 cells is consistent with the conclusion that culturing LPCs in the peptide hydrogel structure under the media and growth factor conditions described above induced a portion of the cells to differentiate along a pathway that leads ultimately to mature hepatocytes. Thus culture within the peptide hydrogel structure rendered the LPCs plastic for instruction (e.g., by growth and/or differentiation factors) to produce cells with either hepatocyte or neuronal properties. The results are consistent with the conclusion that the unique properties of the hydrogel environment permitted this type of transdetermination.

Example 5

Growth Rate Analysis of LPC-8 Cells After Hydrogel Encapsulation

Materials and Methods

Cells, Cell Culture, and Encapsulation. LPC 8.1 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and Xs (400 μM) and monitored for a period of 4 days. Control cells were grown on conventional polystyrene tissue culture dishes. Cells were encapsulated in RAD16-I hydrogel (0.5% w/v) as described above. For some experiments cells were removed from the hydrogels by mechanical disruption and transferred to conventional tissue culture dishes, where they were maintained in the same medium. Since no enzymatic digestion is used, spheroids can be successfully transferred to conventional tissue culture dishes almost entirely intact to facilitate analysis. Cells were counted using a hemacytometer.

BrdU Staining. BrdU staining was performed as in Example 2.

Results

After the first 24 hr of culture, the number of cells in colonies growing on conventional culture dishes (control) increased exponentially with an approximate doubling time of 24 hr. Cell division was quantified by calculating the relative growth ratio (Rgr), where the mean population of cells per colony duplicates every 24 hr. For the control culture, the cell number increased from 5.3±1.8 cell/colony to 11.0±2.0 cell/colony in one day (Rgr= 2.1) and to 40.6±5.4 in 3 days (Rgr=7.7), where the mean population increased by almost eight (8) times (Table 3). In the peptide hydrogel cultures, the cell growth kinetics were very different. Initially, small clusters of 3 to 6 cells were observed after 24h of culture (4.14±1.9 cells/cluster) (Fig. 8a,8b and Table 3). In the following days the clusters continued growing and adopted a spheroid morphology (Fig. 8c, 8d). The average number of cells in the spheroids increased slowly when compared to the control cultures (Table 3). For the hydrogel-cultured spheroids, cell density increased from 4.14±1.9 cells/cluster to 6.33±1.9 cells/cluster after 24 hr (Rgr=1.5), and to 15±3.8 cells/cluster after 3 days (Rgr=3.6) (Table 3). This dramatic difference in the growth kinetics suggested that either the generation time significantly increased in the peptide hydrogel cultures or that only a reduced number of cells per spheroid maintained mitogenic activity while the rest underwent cell cycle arrest, or both (Table 3). The effect of the hydrogel on LPC-8 growth occurred despite the presence of Xs, suggesting a different mechanism of cell division control caused by the interaction with the three-dimensional scaffold.

To study the mitotic activity of the spheroids in greater detail, we labeled dividing cells with the thymidine analog, 5'-bromodeoxyuridine (BrdU), which incorporates into DNA during S-phase of the cell cycle. Cells were labeled for 24 hr (i.e., about 1 generation time) to detect the entire population of dividing cells. Due to the 3-dimensional environment, it was difficult to count the exact number of $BrdU^+$ cells in the spheroids by visual inspection. We thus extracted them from the cultures by mechanical disruption of the hydrogel and transferred them to conventional cell culture dishes for analyses, where they formed a small colony on the cell culture

dish surface (spheroid-colony), suitable for further visual analysis. We compared BrdU incorporation in 48 hr-old conventional culture dish and 96 hr-old peptide hydrogel culture spheroids. As expected, the BrdU-positive ($BrdU^+$) fraction of the cells in the colonies cultured on the dishes was very high (>95%) (Fig. 8e, 8f). In contrast, a reduced fraction of the cells in the spheroid-colonies (<50%) incorporated BrdU, indicating that many cells were arrested after four days in hydrogel cultures (Fig. 8g, 8h). The phenotype of the arrested cells in the spheroid-colonies was unusual; they exhibited an increased cell size with a high incidence of binucleated cells that were often $BrdU^-$ (Fig. 8g, 8h) Blue arrows in Figure 8 indicate binucleated cells.

Table 3. LPC-8 colony growth rate on regular culture plates or in RAD16-I peptide hydrogels[1].

Culture time (hr) (n=8)	Number of Cells per colony or spheroid (Mean±SD)[2]	
	Culture dish (n=3)	RAD16-I hydrogel
24	5.3±1.8	4.14±1.9
48	11.0±2.0 (~2.1)[3]	6.33±1,1 (~1.5)
96	40.6±5.4 (~7.7)	15.0±3.8 (~3.6)

[1]LPC-8 cells were cultured on regular 6-well culture dishes (n=3) and plated at initial density of ~10,000 cells/cm^2 or encapsulated into RAD16-I peptide hydrogels (0.5% w/v) at initial concentration of ~100,000 cell/ml (n=8), contained in culture inserts (see Methods). The media used was DMEM with 10% fetal bovine serum and xanthosine (400 μM) to induce exponential growth of LPC-8 cells. Cells were cultured for 24 hr, and the number of cells per colony was counted by visual inspection under a stereo microscope. [2]Data is expressed as mean value ± standard deviation (SD). [3]Relative growth ratio (Rgr) was calculated after dividing each mean value from 48 hr and 96 hr (Mv_t, where t= 48 hr or 96 hr) by the initial mean value from 24 hr (Mv_i) as follow: $Rgr = Mv_t / Mv_i$.

Example 6

Phenotypic Characterization of Cells Extracted from Hydrogels after Growth in DMEM

Materials and Methods

Cells, Cell Culture and Encapsulation. LPC-8.1 cells were grown in DMEM/10% FBS/pen/strep as described above. Cells were encapsulated in RAD16-I hydrogels as described above.

Cell and Spheroid Extraction from the Hydrogels. LPC-8.1 cells were cultured for 48 hrs

on regular (i.e., conventional) cell culture dishes at a density of ~10,000 cells/cm^2 (controls) or for 96 hrs after encapsulation in RAD16-I hydrogels (0.5% w/v) at a

density of ~100,000 cell/ml. Since encapsulated cells grow more slowly than cells grown on regular cell culture dishes, the number of cells present after culturing for 48 hours on regular plates is approximately the same as the number of cells present after culturing encapsulated cells for 96 hours.

Spheroid-colony isolation from hydrogel cultures. Hydrogel cell cultures were disrupted mechanically with a Pasteur pipette by several up and down aspirations until about 50% of the cells/clusters were extracted as determined by phase microscopy. The suspension was placed on regular culture plates and incubated overnight in the same media at 37 °C equilibrated with 5% CO_2 to allow spheroid-colony formation. The next day, the remaining hydrogel was removing by washing the wells with fresh media and the attached cells/clusters were used for BrdU uptake, CYP1A1/CPY1A2 activities, and immunofluorescence analyses as described above.

Immunostaining. Immunostaining was performed as described above except that 4% paraformaldehyde was used. Primary antibodies used were: rabbit polyclonal anti-rat Cytochrome P450 enzyme CYP1A1 and CYP1A2 (CHEMICON International, catalog number: AB1255), rabbit IgG anti-rat albumin-HRP (Accurate Chemicals, YNGRAALBP), rabbit IgG anti-rat C/EBPα (Santa Cruz Biotechnology, catalog number: sc-61), mouse IgG1 monoclonal anti-rat Cytokeratin 8 (CHEMICON International, Inc. MAB1673), (CHEMICON International, Inc. MAB3226), mouse IgG1 monoclonal anti-Cytokeratin 18 (Santa Cruz Biotechnology, sc-6259), mouse IgG1 monoclonal anti-Cytokeratin 19 (Santa Cruz Biotechnology, sc-6278). Secondary antibodies used were: goat anti-mouse IgG Rhodamine-conjugated (Santa Cruz Biotechnology, catalog number: sc-2029); donkey anti-rabbit Rhodamine-conjugated (Santa Cruz Biotechnology, catalog number: sc-2095).

Measurement of CYP1A1 Activity. Measurement of CYP1A1 activity was performed essentially as described above.

Light Microscopy and Photography. Light microscopy and photography were performed as described above.

Results

The data presented above suggested that the presence of various growth factors in the growth environment influences the differentiation and transdifferentiation properties of cells cultured in the hydrogels. To further explore this phenomenon LPC-8 cells were cultured in DMEM/FBS either on conventional tissue culture dishes (control) or in RAD16-I peptide hydrogels for either 48 hours (control) or 96 hours (encapsulated). At this time the cell numbers were approximately equivalent. Cells were then removed from their respective culture environment either by trypsinization (for control cultures) or by mechanical disruption (for spheroids isolated from hydrogels). Cells were then cultured in the same medium overnight on conventional tissue culture dishes to allow spheroid colonies to form. Spheroids were then immunostained for expression of various markers.

Figure 9 shows phenotypic analysis of LPC cells during exponential growth on conventional culture dish either after isolation from regular culture conditions or after isolation from peptide hydrogel culture. Figures 9a, 9c, 9e, 9g, 9i, and 9k show phase contrast micrographs of control or spheroid-derived colonies. Figures 9b, 9d, 9f, 9h, 9j, and 9l show the same colonies immunostained to show expression of C/EPBα, albumin, or CYP1A1/1A2. Table 4 presents a semi-quantitative comparison of expression of these and the additional markers α-fetoprotein, cytokeratin 18 (CK18), and cytokeratin 19 (CK19) in control and spheroid-derived colonies. Table 4 also compares the CYP1A1 activity and the number of binucleated cells (consistent with a hepatocyte phenotype) in control and spheroid-derived colonies.

The expression of α-fetoprotein did not change after culturing LPC-8 cells in hydrogels, suggesting that the treatment did not affect the expression of the fetal marker (Table 4). In contrast, the developmental marker C/EBPα was highly expressed in most of the nucleus of 2 and 10 days-old spheroid-derived colonies compared to the expression in control colonies. This provides evidence that LPC-8 cells encapsulated in hydrogels initiate differentiation along a pathway consistent with hepatic lineage cells (Fig. 9a-9d and Table 4). Other hepatic markers such as

albumin and CK18 were upregulated in the spheroid-colonies (Fig.9e-9h and Table 4). Albumin expression was clearly visible in the cytoplasm, as expected (Fig. 9h). Similar levels of CK8 were observed both in cells from control colonies and cells from colonies grown in hydrogels, indicating the hepatocyte lineage of the cells. However, cells from control colonies and hydrogel-derived colonies were negative for CK7 and CK19 (Table 4), suggesting that Lig-8 are not from oval cell origin or differentiating into biliary duct epithelium during the time of experiment. These results suggested that spheroid-colonies displayed a clear hepatocyte phenotype because no biliary duct epithelium marker (CK7; CK19) was observed (Tables 4 and 6). The presence in LPC-8 hydrogel-derived spheroid-colonies of the common cellular markers expressed in hepatocytes as well as hepatic oval cells such as albumin, CK18, and α-fetoprotein (Fig 9 and Table 4) is consistent with the interpretation that the liver stem cell LPC-8 is progressively differentiating in the hydrogels into hepatocytes through an oval cell intermediate as suggested previously for adult hepatic differentiation. Nevertheless, the absence of expression of CK19 (a rat hepatic oval cell and biliary duct epithelium marker) in any of the culture conditions tested, the high expression in hydrogel-derived spheroid colonies of C/EBPα, which is mainly expressed in hepatocytes (Fig 9 and Table 4) and the increasing presence of binucleated cells and expanded cell body (a common phenotype found in hepatocytes) suggests that a terminal differentiation process into hepatocytes may be occurring. Moreover, the majority of the binucleated cells present in the hydrogel-derived spheroid-colonies were also BrdU⁻, suggesting that they were post-mitotic differentiated cells (Fig.8, Fig 9 and Table 4). Blue arrows in Figure 9 indicate binucleated cells.

The expression of cytochrome P450 1A1 and 1A2 enzymes (CPY1A1 and CYP1A2) was also up-regulated dramatically in hydrogel-derived spheroid colonies indicating the presence of a potential hepatic function in the hydrogel cultures (Fig. 9i-9l, Table 4). In view of these results we tested activity of CYP1A1 in control and hydrogel cultures over a period of two weeks. Low CYP1A1 activity (0.02±0.01 pmol/cell/hr) was detected in exponentially growing cells on conventional culture dishes (Table 4). However, after only two days of hydrogel culture, an increase in CYP1A1 activity (0.09±0.01 pmol/cell/hr) was observed. This activity was

maintained for 10 days (0.09-0.01 pmol/cell/hr), and for up to 2 weeks (0.08±0.01 pmol/cell/hr) (Table 4). This 3 to 4 fold increase in CYP1A1 activity correlates well with the increase in expression of CYP1A1 and CYP1A2 protein (Fig. 9i-9l). Overall, inventors suggest that the results described above indicate that LPC-8 is a clonal, somatic (liver-derived) stem cell that can be instructed to differentiate into a hepatocyte lineage by culturing in a nanoscale environment comprising nanoscale fibers. In particular, inventors suggest that the results described here indicate that LPC-8 cells can be instructed to differentiate into a hepatocyte lineage by culturing them in a three-dimensional nanofiber environment comprising a self-assembling peptide hydrogel. As described in previous examples, under the influence of various agents such as growth factors, LPC-8 cells may also be instructed to differentiate into neural lineages by culturing them in a nanoscale environment comprising nanoscale fibers, e.g., by culturing them in a three-dimensional nanofiber environment comprising a self-assembling peptide hydrogel.

Table 4. Phenotypic characterization of LPC-8 cells during exponential growth in regular culture conditions (2D-culture) or after RAD16-I peptide hydrogel cultures (3D-culture).

Cellular marker[1]	Dish culture (2D)[2] (48 hr)	RAD16-I hydrogel (3D) (48 hr)	(240 hr)
α-Fetoprotein	+[3]	+	+
C/EBPα	-	+++	+++
Albumin	-/+	+++	+++
CK18	-	+	+
CK19	-	-	-
CYP1A1 activity[4]	0.02±0.01	0.09±0.01	0.09-0.01
CYP1A1/CYP1A2	-/+	+++	+++
Binucleated cells	-	+	++

[3]Quantification in terms of marker expression or phenotype: (-), not detected; (-/+), very low; (+), low; (++), medium; (+++), high. [2]Regular polystyrene cell culture dish. [4]CYP1A1 activity was detected by measuring release of resofurin after incubating the cultures in presence of 7-ethoxyresofurin (see Materials and Methods). [1]α-fetoprotein, present in fetal hepatocytes and hepatic oval cells; C/EBPα, marker for hepatocytes, intestinal epithelial cells, and fat cells; albumin, marker for hepatocytes and hepatic oval cell; CK18, expressed in hepatocytes, biliary duct epithelium and hepatic oval cells; CK19, expressed in biliary duct epithelium and hepatic oval cell; CYP1A1/CYP1A2, present in hepatocytes and other cells; Binucleated cells, common phenotype in hepatocytes.

Example 7

CYP1A1 and CYP1A2 Activity in Cells Following Culture in Peptide Hydrogels

Materials and Methods

Cells, Cell Culture and Encapsulation. LPC-8.1 cells were grown in DMEM/10% FBS/pen/strep as described above. Cells were encapsulated in RAD16-I hydrogels as described above. LPC-8.1 cells were cultured on regular (i.e., conventional) cell culture dishes at a density of ~10,000 cells/cm^2 (controls) or after encapsulation in RAD16-I hydrogels (0.5% w/v) at a density of ~100,000 cell/ml.

Spheroid Isolation from Peptide Hydrogels. Hydrogel cell cultures were disrupted mechanically with a Pasteur pipette by several up and down aspirations until about 50% of the cells/clusters were extracted as determined by phase microscopy. The suspension was placed on regular culture plates and incubated overnight in the same media at 37 °C equilibrated with 5% CO_2 to allow spheroid-colony formation. The next day, the remaining hydrogel was removing by washing the wells with fresh media and the attached cells/clusters were used for assessment of CYP1A1/CPY1A2 activities.

Cytochrome P450 induction by 3-methylcholanthrene (3-MC). The aromatic hydrocarbon 3-MC (Sigma) was added to the culture media of control and hydrogel cultures at a final concentration of 2 μM starting on the fourth (4^{th}) day of culture. The media containing 3-MC was replaced (50%) every other day.

Cytochrome P450 1A1 and 1A2 activities. Cytochrome P450 1A1 (CYP1A1) and Cytochrome P450 1A2 (CYP1A2)-dependent *0*-dealkylation activities on a resorufin alkyl ether substrate (7-ethoxyresorufin and 7-methoxyresofurin, respectively) were analyzed over time in LPC-8 cells growing on regular culture plates and in hydrogel derived LPC spheroid colonies isolated and cultured as described above using an *in situ* assay (Donato, M.T., Gomez-Lechon, M.J. & Castell, J.V., A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates, *Anal. Biochem.* 213, 29 (1993)). The reaction analyzed was the 7-ethoxyresorufin *0*-deethylation (EROD) and 7-methoxyresofurin *0*-demethylation (MROD) activities that release resorufin as product. The incubations (triplicates) were carried out for 15 min at 37°C in the presence of 3 μM of 7-ethoxyresorufin or 3 μM of 7-methoxyresorufin and 10 μM of dicumarol (Sigma) that prevents the decay of resorufin due to further metabolism by cytosolic oxidoreductases. The resorufin product was measured by the increase in the fluorescence intensity using excitation/emission filters of 530/590 nm, respectively. Resorufin product concentration was calculated based on a known resorufin standard curve obtained using the same culture media. The CYP activity was expressed in units of pmol resorufin produced/cell/hour.

In vivo metabolism of caffeine-8-[^{14}C]. Control or hydrogel cultures treated or not with 3-methylcholantrene (3-MC) for 48hr were incubated for additional 24hr in presence of 0.4 mM of caffeine-8-[^{14}C] (~2 μCi of 53 mCi/mmol in 100 μL culture). After the incubation, the samples were boiled for 4 min at 100 °C to stop the reaction and sonicated for 5 min to facilitate solubilization of the reaction products. The samples were centrifuged at 14,000 rpm and an aliquot of 5 μL (5%) were loaded on a thin layer chromatography (TLC) plate (silica gel 60, F254 ENSCIENCE) with external standards (Sigma) of caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), theobromide (3,7-dimethylxanthine), paraxanthine (1,7-dimethylxanthine), 3-methylxanthine, xanthine. The plate was developed using the organic solvent mix chloroform/acetone (1:1) as a mobile phase. External standards were visualized by exposing to UV light (254 nm) and the radioactive compounds by exposure overnight to a X-film (Kodak Biomax film). Radioactive compounds detected with the same relative mobility as paraxanthine were extracted from the TLC and counted for radioactivity in a scintillation counter. The amount of paraxanthine produced was calculated and expressed in pmol/day/10^4 cells.

Results

The activity of CYP1A1 and CYP1A2 in control and hydrogel-derived LPC spheroid colonies over a ten day period was measured using a fluorometric method. Figure 10 presents the time course of CYP1A1 and CYP1A2 activity in control cultures (Fig. 10a and 10b) and hydrogel derived cultures (Fig. 10c and 10d) either in the presence (squares) or absence (circles) of treatment with the polycyclic aromatic hydrocarbon 3-MC starting at a time indicated by the arrow. Untreated control cultures exhibited only weak CYP1A1 activity, and activity of CYP1A2 activity was undetectable (Fig. 10a and 10b). After only four days, the CYP1A1 activity in untreated spheroid colonies exceeded that in the control by 3 to 4-fold, and activity continued to increase during the ten-day experimental period (Fig. 3d). CYP1A2 activity also increased to a detectable level in untreated spheroid colonies remained stable for the longest period of evaluation, 10 days (Fig. 10d).

In order to determine whether CYP1A1 and CYP1A2 expression in the hydrogel system is likely to be under the transcription regulation of the Ah receptor

(AHR) control and hydrogel derived cultures were incubated in the presence of 3-methylcholanthrene (3-MC), which is known to induce CPY1A1 and CYP1A2 expression through the ligand-activated transcription factor AHR (Burbach, et al.). As shown in Figure 10, both CYP activities were induced by 3-MC treatment in the hydrogel derived spheroid colonies (Fig. 10c and 10d) but not in control cultures (Fig. 10a and 10b). In the hydrogel derived cultures CYP1A1 and CYP1A2 activities were increased by about 100% and 300%, respectively, relative to the activity in non-induced samples.

Caffeine (1,3,7-trimethylxanthine) was used to analyze the CYP1A2 activity of the hydrogel cultures because it has previously been described that CYP1A2 specifically catalyzes the 3-*N*-demethylation of caffeine to produce paraxanthine (1,7-dimethylxanthine) (Butler, M.A., Iwasaki, M., Guengerich, F.P. & Kadlubar, F.F. Human cytochrome P-450PA (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc. Natl. Acad. Sci. USA* 86, 7696-7700 (1989)). Radiolabelled caffeine (caffeine-8-[^{14}C], 0.4 mM) was added to control and hydrogel cultures, with or without exposure to 3-MC, and incubated *in vivo* for 24hr. Paraxanthine-8-[^{14}C] product obtained was detected after separating samples of total cell culture extracts by thin layer chromatography (TLC). Table 5 presents a summary of the data. As expected, caffeine 3-*N*-demethylating activity was evidenced by the appearance of detectable levels of paraxanthine-8-[^{14}C] in hydrogel cultures but not in controls (Table 5). Furthermore, hydrogel cultures exposed to 3-MC displayed approximately a three to four fold greater amount of the product paraxanthine relative to uninduced cultures (Table 5), suggesting again that the increment in CYP1A2 activity observed might be due to the ligand-activated transcription factor AHR. These results suggest the presence of functional Ah receptors on the hydrogel-derived spheroid colony cell membranes.

Table 5. Caffeine[1] biotransformation by CYP1A2 in Lig-8 hydrogel and control cultures after induction with 3-methylcholantrene (3-MC).

Metabolite detected	Control culture		Hydrogel	
culture	- 3-MC	+ 3-MC	- 3-MC	+ 3-MC
Paraxanthine[2]	< 1.8	< 1.8	27.2	101.8

[1] Caffeine (caffeine-8-[^{14}C], 0.4 mM) was incubated in vivo in control and hydrogel cultures exposure or not to 3-methylcholanthrene (3-MC).

[2] Amount of paraxanthine obtained is expressed in pmol/day/10^4 cells.

Table 6. Hepatic phenotype of LPC cultures before and after RAD16-I peptide hydrogel treatment

Marker[3]	Untreated cells	Cultured in RAD-16 hydrogel[2]	
	(48 hr)	(48 hr)	(240 hr)
Fetal hepatocyte			
α-Fetoprotein	+[1]	+	+
Mature hepatocyte			
C/EBPα	-	+++	
Albumin	-/+	+++	
CK8	+	+	+
CYP1A1/CYP1A2	-/+	++	
Functional hepatocyte			
3-MC inducible CYP1A1	-	+	
3-MC inducible CYP1A2	-	++	
Hepatocyte morphology			
Binuclear cells	-	+	
Biliary duct epithelium			
CK7	-	-	-
CK19	-	-	-

[1]Quantification terms for marker expression: (-), not detected; (-/+), very low; (+), low; (++), medium; (+++), high expression. [2]Cells were either grown in standard adherent culture without peptide hydrogel exposure; or cultured for the indicated times in peptide hydrogels and then transferred to adherent culture for analysis as described in the Examples. [3]α-fetoprotein, present in fetal hepatocytes and hepatic oval cells; C/EBPα, marker for hepatocytes, intestinal epithelial cells, and fat cells; albumin production and secretion, marker for mature hepatocytes; CK8, marker for hepatocytes and biliary duct epithelium; CK19, marker for oval cells and biliary duct

epithelium; CYP1A1/CYP1A2, present in hepatocytes; as well as their induction by 3-methylcholantrene (3-MC), and binuclear cells is a mature hepatocyte phenotype.

Example 8

Differentiation of Liver Progenitor Cells into Insulin-Secreting Beta-like Cells

Materials and Methods

Encapsulation of LPCs in hydrogel: LPC-8 cells were encapsulated in RAD16-I hydrogel (~100,000 cells/well) in multi-well inserts (0.5 mm diameter) so that media could diffuse through evenly from the bottom essentially as described in Example 1 except that for experiments involving fibronectin or laminin, either fibronectin (100 μg/ml) or laminin (100 μg/ml) was added to the cell suspension prior to mixing with the hydrogel solution. Thus each insert contained 50 μL of cell suspension in 10% sucrose (w/v) in the absence or presence of fibronectin (50 μg/ml) or laminin (50 μg/ml); this suspension was mixed with 50 μL of hydrogel solution (0.5% final concentration of peptide) by pipetting up and down several times. Regular culture media (DMEM, high-glucose, 25 mM, without sodium pyruvate/10% FCS/pen-strep) containing EGF (0.8 ng/ml), FGF-2 (0.5 ng/ml), or a mix of both (0.8 ng/ml EGF and 0.5 ng/ml FGF-2) was added to the well bottom (0.5 ml) in order to contact the insert membrane and initiate hydrogel formation. (FGF-2 is also referred to as basic FGF, FGFβ, or βFGF). Unless otherwise stated all medium used in the experiments in this Example included 10% FCS/pen/strep.

Differentiation protocol. Cells in hydrogels were maintained in tissue culture inserts in the DMEM, high glucose (25 mM) medium with or without growth factors at the concentrations described immediately above. Medium (50%) was changed every other day, with the addition of fresh growth factors. Cells were monitored daily. After two weeks (14 days), the high-glucose media was replaced by low-glucose media (DMEM, 5 mM glucose, without sodium pyruvate) containing nicotinamide (final concentration 10 mM) but without FGF-2 or EGF. The cultures were incubated for an additional 48 hr. Then, the hydrogels were mechanically disrupted, and the cell clusters were plated on laminin-coated 6-well dishes (Becton and Dickinson) overnight in low glucose (5 mM) DMEM medium without EGF, FGF-2, or nicotinamide. The next day, the cluster colonies formed were used for

characterization and analysis of insulin secretion. The total time of the differentiation protocol optimized for formation of islet-like structures was thus equal to 17 days, but shorter culture periods also resulted in formation of islet-like structures.

Immunofluorescent analysis: Immunostaining was used to detect the presence of insulin in cells. After encapsulation in hydrogels, incubation, subsequent disruption of hydrogel cultures, and overnight plating in DMEM to allow colony formation, cells were fixed with 2% paraformaldehyde in PBS for 2 hours, washed with PBS, treated with 0.1% Triton X-100 in PBS for 2 hours, and incubated in blocking buffer (20% calf serum, 0.1% Triton X-100; 1% DMSO in PBS) for 4 hours at room temperature. The cells were then treated with anti-insulin mouse monoclonal antibody (Sigma) overnight, followed by anti-mouse IgG FITC-conjugated for detection under a Nikon microscope TE300.

Induction of insulin release by glucose and other agents: To detect and quantify insulin release in response to glucose induction, cells were cultured in hydrogels under optimum differentiation conditions (two weeks in hydrogel containing laminin; DMEM with high glucose, EGF + FGF with switch to low glucose DMEM with nicotinamide for the final two days followed by spheroid extraction and plating overnight on laminin-coated dishes). The plated cells were cultured in DMEM (5 mM glucose) and then incubated at 37°C with different concentrations of glucose (from 0 to 40 mM) for 10 min in Krebs-Ringer buffer (NaCl, 120 mM; KCl, 5 mM; $CaCl_2$; 2.5 mM; $MgCl_2$, 1.1 mM; $NaHCO_3$, 25 mM; BSA, 0.1%) at 37°C. In addition, cells were incubated in Krebs-Ringer buffer in the presence of other agonists of insulin release such as carbachol (100 mM; Sigma), 3-isobutil-1-methylxanthine (IBMX) (100 mM; Sigma), and tolbutamide (10 mM; Sigma), in conjunction with low glucose (5 mM). After incubation, the supernatant was removed and stored for later analysis. The cells were either fixed in paraformaldehyde for staining, incubated in lysis buffer for total protein quantitation, or extracted with acetic acid/ethanol solution (10% acetic acid in ethanol) to extract intracellular insulin content. An ELISA assay specific for rat

insulin detection (Alpco) was used to analyze the supernatant for insulin release and the cell extract for cellular insulin content. The assay was performed according to the directions of the manufacturer. Total protein levels were determined using a protein detection kit (Bio Rad) to normalize insulin amounts according to total protein levels in each well. Assays were performed in triplicate and standard deviation was calculated. Analysis of intracellular insulin content may be performed using a protocol involving extraction with acetic acid/ethanol solution (10% acetic acid in ethanol) as described Lumelsky, et al., 2001.

Analysis of cells grown in 2-D conditions: To verify that three-dimensional conditions are required for cell differentiation, LPC-8 cells were also cultured on laminin- and fibronectin-coated plates (Becton & Dickinson) in media containing EGF and FGF-2 at the same concentrations as used for hydrogel cultures. They were subsequently stained for insulin expression.

Results

Cells cultured in hydrogels with ECM components form distinctive islet-like structures. The appearance of cells cultured in hydrogels in DMEM high glucose with different combinations of EGF and/or FGF and in the presence or absence of fibronectin or laminin was monitored daily. Formation of distinctive clusters was generally evident in the cultures containing fibronectin and laminin within one week of encapsulation, and such clusters were consistently observed within two weeks. The clusters differed in both size and structure from clusters observed under conditions favoring differentiation into hepatocyte-like cells (e.g., DMEM/10% FBS/pen/strep as described in Examples above). In particular, in the presence of fibronectin or laminin (ECM+) the clusters were much larger, contained many more cells, and exhibited a more well defined structure than in the absence of these ECM components. The ECM+ clusters contained a flat layer of cells on the surface that cover a group of approximately 50-100 cells in the interior. Figure 11 compares control clusters grown in peptide hydrogels in the absence of growth factors and ECM components (Fig. 11a, 11b) and clusters grown in peptide hydrogels in the presence of laminin, EGF, and FGF-2 (Fig. 11c, 11d). Clusters were grown for 8 days in both cases. The larger size and more organized structure of the clusters in Fig. 11c and 11d is readily apparent. Arrows indicate the surface cells.

Insulin production by cells in hydrogel culture. After one to two weeks in hydrogel culture the medium was switched to low glucose medium with nicotinamide but without growth factors, and cells were cultured for an additional two days. Clusters were isolated from the hydrogels and plated on laminin-coated regular tissue culture dishes overnight to allow colony reformation. Immunostaining the colonies with an anti-insulin antibody demonstrated that colonies grown in the presence of laminin with EGF, FGF-2 or both or in the presence of fibronectin with EGF, expressed high numbers of insulin granules. Colonies grown in hydrogels in the absence of laminin, fibronectin, and/or EGF did not show insulin staining. Similarly, insulin was not detected in colonies cultured on laminin or fibronectin coated plates in identical medium without having been precultured in the peptide hydrogel. Thus either EGF, laminin, or fibronectin in the context of the hydrogel appears to be sufficient to induce formation of insulin producing cells while FGF-2 alone is insufficient. optimized for formation of islet-like structures was thus equal to 17 days, but shorter culture periods also resulted in formation of islet-like structures. Culture for two weeks in hydrogels in the presence of EGF, FGF-2, high glucose, and laminin followed by switching to low glucose medium with the addition of nicotinamide for the final two days appeared optimum and consistently produced insulin secreting islet-like structures. High glucose appears to be necessary for the differentiation and proliferation phase of the protocol.

Table 7 summarizes results obtained using an 8 day differentiation protocol and also indicates relative number of colonies that stained positive for insulin. Figure 12 shows insulin staining of a control colony grown in the absence of ECM components and growth factors (Fig. 12b), a colony grown in the presence of fibronectin and EGF (Fig. 12d), and a colony grown in the presence of laminin and EGF + FGF-2 (Fig. 12f). Brightly staining insulin granules are readily visible in Figures 12d and 12f but are absent in Figure 12b.

Table 7. Detection of insulin granules by immunofluorescence.

	None	Laminin (50 μg/mL)	Fibronectin (50 μg/mL)
None	-	-	-
EGF (0.8 ng/mL)	+	+	++
FGFb (0.5 ng/mL)	-	+	-
EGF + FGFb	+	++	+

Spheroid-colonies prepared from different hydrogel culture conditions presented in the Table were immunostained using a mouse monoclonal antibody anti-rat insulin. The amount of granules in each case is expressed as: -, no detected presence of insulin granule-containing cells in spheroid-colonies; +, presence of insulin granules in approximately 25% of cells per spheroid-colony; ++, presence of insulin granules in approximately 50% of cells per spheroid-colony.

Glucose induces insulin release by hydrogel derived pancreatic β-cell-like colonies.

After one to two weeks in hydrogel culture the medium was switched to low glucose medium with nicotinamide but without growth factors, and cells were cultured for an additional two days. Clusters were isolated from the hydrogels and plated on laminin-coated regular tissue culture dishes overnight to allow colony reformation. Cells were then exposed to a variety of different compounds known to stimulate insulin secretion. These included glucose (0 to 40 mM); carbachol, an agonist of muscarinic cholinergic receptors; 3-isobutil-1-methylxanthine (IBMX), an inhibitor of cyclic-AMP phosphodiesterase; and tolbutamide, a sulfonylurea inhibitor of ATP-dependent K+ channel. The latter three compounds were used in conjunction with low glucose (5 mM). Supernatant was analyzed to measure insulin release.

Figure 13 compares insulin release by cells exposed to various stimulants. Assays were performed in triplicate and error bars represent standard deviation. As indicated in Figure 13, cells cultured in higher concentrations of glucose released higher levels of insulin, with a background release of approximately 8-11 ng of insulin/mg protein at 0 and 5 mM glucose, respectively, and a higher release of approximately 37 ng of insulin/mg protein at 10 mM glucose concentration. Spheroid clusters grown in hydrogels without either laminin, fibronectin, or growth factors released a small amount of insulin (approximately 3-4 ng/mg protein) at low glucose concentrations (0 or 5 mM) and do not respond to increased glucose with an increase in insulin release. Thus these cells do not demonstrate the glucose-

stimulated insulin release characteristic of islet cells. The dramatic response to the change in glucose concentration reflects a physiological behavior of the hydrogel derived pancreatic β-cell-like cells.

As shown in Figure 13, concentrations of glucose on the order of 20 to 40 mM induced approximately equivalent insulin secretion to 10 mM glucose. IBMX and carbachol induced as effectively as glucose, suggesting that either cyclic-AMP/PKA and muscarinic cholinergic receptors through DAG/PKC pathways are also regulating insulin secretion independently of glucose in the hydrogel derived pancreatic β-cell-like system. Cells grown in this system produce about ten times more insulin per mg of protein than that previously reported using embryonic stem cells (Lumelsky, N. *et al.*, *Science* 292, 1389-1394, 2001), (although still five times less insulin than normal islet cells). Adult rat hepatic oval cells (Yang, L. *et al.*, *Proc. Natl. Acad. Sci. USA* 99, 8078-8083, 2002), can apparently differentiate into insulin-secreting structures similar to pancreatic islets. Nevertheless, hepatic oval cells are not a good source of cells for tissue engineering because they must generally be obtained by hepatic chemical injury with 2-acetylamino-fluorene. This procedure is not satisfactory for modern bioengineering, which requires stem cells to be isolated for human patients without harmful procedures.

Insulin secretion in the system described herein occurs in response to physiologically relevant changes in glucose concentrations, in the same way as normal cells from the pancreatic islets respond *in vivo*. Secretion levels may be increased by modulating culture conditions (e.g., by systematically altering ECM components and concentrations, growth factor concentrations and combinations, glucose concentration of medium, etc.). The functional activity of the insulin can readily be tested using assays known in the art. In addition, *in vivo* rescue of insulin-deficient animal models such as the diabetic NOD-*scid* mouse following implantation of insulin-secreting cells can be demonstrated. Adult stem cells may prove to be a valuable source of functional pancreatic islets and other pancreatic cell types for treatment of diabetes and other disorders affecting the pancreas, as they can provide an abundant source of immunocompatible cells and do not introduce the same ethical concerns as embryonic stem cells or difficulties associated with

isolation of oval cells. Adult stem cells may thus represent a more reliable source of a wide variety of differentiated cell types useful in biomedicine and biotechnology.

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CLAIMS

What is claimed is:

1. A macroscopic structure comprising:

amphiphilic peptides, wherein the peptides comprise substantially equal proportions of hydrophobic and hydrophilic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure; and

progenitor cells.

2. The macroscopic structure of claim 1, wherein the progenitor cells are encapsulated in the structure.

3. The macroscopic structure of claim 2, further comprising a differentiation-enhancing agent.

4. The macroscopic structure of claim 1, 2, or 3, wherein the progenitor cells are cells obtained or derived from the liver.

5. The macroscopic structure of claim 1, 2, or 3, wherein at least one of the progenitor cells is a stem cell, which stem cell may be either an adult stem cell or an embryonic stem cell.

6. The macroscopic structure of claim 3, wherein the density of the encapsulated cells is approximately 10^5 cells/ml.

7. The macroscopic structure of claim 3, wherein the density of the encapsulated cells is between 5×10^4 and 5×10^5 cells/ml.

8. The macroscopic structure of claim 3, wherein at least one of the progenitor cells is genetically modified.

9. The macroscopic structure of claim 3, wherein the differentiation-enhancing agent causes a portion of the progenitor cells or their progeny to transdifferentiate.

10. The macroscopic structure of claim 3, wherein the macroscopic structure renders at least a portion of the progenitor cells permissive for instruction by the differentiation-enhancing agent.

11. The macroscopic structure of claim 3, wherein the differentiation-enhancing agent is a growth factor or an extracellular matrix component.

12. The macroscopic structure of claim 11, wherein the growth factor is selected from the group consisting of epidermal growth factor, nerve growth factor, transforming growth factor-β, platelet-derived growth factor, insulin-like growth factor, acidic fibroblast growth factor, basic fibroblast growth factor, hepatocyte growth factor, brain-derived neurotrophic factor, keratinocyte growth factor, bone morphogenetic protein, or a cartilage-derived growth factor.

13. The macroscopic structure of claim 12, wherein the growth factor is epidermal growth factor, nerve growth factor, or basic fibroblast growth factor.

14. The macroscopic structure of claim 3, wherein the progenitor cells are derived through a process including suppression of asymmetric cell kinetics prior to encapsulation.

15. The macroscopic structure of claim 3, wherein the progenitor cells include non-neuronal cells, and wherein at least a portion of the progenitor cells or their progeny express a neuronal marker following encapsulation and exposure to the differentiation-enhancing agent.

16. The macroscopic structure of claim 3, wherein the differentiation-enhancing agent is added to a peptide or electrolyte solution or to tissue culture medium prior to self-assembly of the structure.

17. The macroscopic structure of claim 3, wherein the differentiation-enhancing agent is present in or added to tissue culture medium in which the structure is incubated following self-assembly of the structure.

18. The macroscopic structure of claim 1, 2, or 3, wherein the peptide is RAD16-I.

19. The macroscopic structure of claim 1, 2, or 3, wherein the peptides are dissolved in a solution substantially free of electrolytes at a concentration of approximately 0.5% weight/volume prior to self-assembly, or wherein the final concentration of the peptides following self-assembly is between 1 and 10 mg/ml, inclusive.

20. The macroscopic scaffold of claim 3, wherein at least a portion of the progenitor cells or their progeny differentiate or transdifferentiate into cells expressing a marker for mature hepatocytes.

21. The macroscopic scaffold of claim 3, wherein at least a portion of the progenitor cells or their progeny differentiate or transdifferentiate into cells expressing a marker for mature hepatocytes and at least a portion of the progenitor cells or their progeny differentiate into cells expressing a neuronal marker.

22. A method of culturing cells comprising:

providing progenitor cells;

contacting the progenitor cells with a cell culture material comprising amphiphilic peptides, wherein the peptides comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure.

23. The method of claim 22, wherein the contacting comprises placing the progenitor cells on the surface of the material.

24. The method of claim 22, wherein the contacting comprises encapsulating the progenitor cells in the material.

25. The method of claim 24, wherein the step of encapsulating comprises:

(a) incubating the peptides and the progenitor cells in an aqueous solution comprising an iso-osmotic solute; and

(b) adding an electrolyte to the solution sufficient to initiate self-assembly of the peptides into a beta-sheet macroscopic structure, whereby the cells are encapsulated by the formation of the scaffold.

26. The method of claim 24, further comprising adding a differentiation-enhancing agent either before or after assembly of the structure.

27. The method of claim 26, wherein the progenitor cells are cells obtained or derived from the liver.

28. The method of claim 26, wherein at least one of the progenitor cells is a stem cell, which stem cell may be either an adult stem cell or an embryonic stem cell.

29. The method of claim 26, wherein the density of the encapsulated cells is approximately 10^5 cells/ml.

30. The method of claim 26, wherein the density of the encapsulated cells is between 5×10^4 and 5×10^5 cells/ml.

31. The method of claim 26, wherein at least one of the progenitor cells is genetically modified.

32. The method of claim 26, wherein the differentiation-enhancing agent causes a portion of the progenitor cells or their progeny to transdifferentiate.

33. The method of claim 26, wherein the macroscopic structure renders at least a portion of the progenitor cells permissive for instruction by the differentiation-enhancing agent.

34. The method of claim 26, wherein the differentiation-enhancing agent is a growth factor or extracellular matrix component.

35. The method of claim 34, wherein the growth factor is selected from the group consisting of epidermal growth factor, nerve growth factor, transforming growth factor-β, platelet-derived growth factor, insulin-like growth factor, acidic fibroblast growth factor, basic fibroblast growth factor, hepatocyte growth factor, brain-derived neurotrophic factor, keratinocyte growth factor, bone morphogenetic protein, or a cartilage-derived growth factor.

36. The method of claim 35, wherein the growth factor is epidermal growth factor, nerve growth factor, or basic fibroblast growth factor.

37. The method of claim 26, wherein the progenitor cells are derived through a process including suppression of asymmetric cell kinetics prior to encapsulation.

38. The method of claim 26, wherein the progenitor cells include non-neuronal cells, and wherein at least a portion of the progenitor cells or their progeny express a neuronal marker following encapsulation and exposure to the differentiation-enhancing agent.

39. The method of claim 26, wherein the differentiation-enhancing agent is added to a peptide or electrolyte solution or to tissue culture medium prior to self-assembly of the structure.

40. The method of claim 26, wherein the differentiation-enhancing agent is present in or added to tissue culture medium in which the structure is incubated following self-assembly of the structure.

41. The method of claim 22, 24, or 26, wherein the peptide is RAD16-I.

42. The method of claim 24, wherein the peptides are dissolved in a solution substantially free of electrolytes at a concentration of approximately 0.5% weight/volume prior to self-assembly, or wherein the final concentration of the peptides following self-assembly is between 1 and 10 mg/ml, inclusive.

43. The method of claim 26, wherein at least a portion of the progenitor cells or their progeny differentiate or transdifferentiate into cells expressing a marker for mature hepatocytes.

44. The method of claim 26, wherein at least a portion of the progenitor cells or their progeny differentiate or transdifferentiate into cells expressing a marker for mature hepatocytes and at least a portion of the progenitor cells of their progeny differentiate into cells expressing a neuronal marker.

45. The method of claim 26, 31, or 32, further comprising extracting cells from the macroscopic structure.

46. The method of claim 45, further comprising culturing the extracted cells *in vitro*.

47. The method of claim 46, further comprising administering the extracted cells to a subject.

48. The method of claim 47, wherein the progenitor cells are derived from the individual.

48. The method of any of claims 22, 24, 26, 31, or 32, further comprising introducing the macroscopic structure into an individual.

49. The method of claim 48, wherein the progenitor cells are derived from the individual.

50. A method of treating an individual comprising:

identifying an individual in need of treatment; and

administering cells to the individual, wherein the cells have been induced to differentiate or transdifferentiate by culturing them encapsulated in a cell culture material comprising amphiphilic peptides, wherein the peptides comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure, and wherein the cells are exposed to a differentiation-enhancing agent.

51. A method of treating an individual comprising:

identifying an individual in need of treatment;

administering cells within the macroscopic structure of any of claims 1, 2, 3, 8, or 9 to the individual.

52. The method of claim 51, in which the cells are extracted from the macroscopic structure prior to administration to the individual.

53. The method of claim 51, wherein the cells remain encapsulated within the macroscopic structure for administration.

54. A cell culture kit comprising:

(a) amphiphilic peptides, wherein the peptides comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure; and

(b) instructions for initiating self-assembly of the peptides into a macroscopic structure.

55. The kit of claim 54, further comprising at least one element selected from the group consisting of: a population of cells, cell culture medium, a predetermined amount of a growth factor, a predetermined amount of an electrolyte, instructions for encapsulating cells within a peptide hydrogel structure and for other uses of the system, instructions for inducing cells to differentiate or transdifferentiate within the scaffold, a vessel in which the encapsulation may be performed, a liquid in which the peptide can be dissolved, an electrolyte for initiating peptide self-assembly, medium for tissue culture, and one or more differentiation-enhancing agents.

56. An assay system comprising:

a population of cells derived from the liver or their progeny, wherein the cells express a cytochrome P450 enzyme, and wherein the cells are encapsulated in a beta-sheet macroscopic scaffold comprising peptides that comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure.

57. The assay system of claim 56, further comprising:

a substrate for the cytochrome P450 enzyme.

58. A method of testing a compound comprising steps of:

contacting a population of cells derived from the liver or their progeny, wherein the cells express a cytochrome P450 enzyme, and wherein the cells are encapsulated in a beta-sheet macroscopic scaffold comprising peptides that comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure, with the compound;

measuring activity of the cytochrome P450 enzyme; and

comparing the level of activity of the enzyme in the presence of the compound with level of activity in the absence of the compound.

59. A method of culturing cells comprising the step of:

encapsulating the cells in a three-dimensional nanoscale environment scaffold.

60. The method of claim 59, further comprising the step of:

maintaining the encapsulated cells in culture.

61. The method of claim 59 or claim 60, further comprising the step of removing the encapsulated cells from the three-dimensional nanoscale environment scaffold.

62. The method of claim 59, wherein the nanoscale environment scaffold comprises a protein or peptide hydrogel.

63. The method of claim 62, wherein the hydrogel comprises a self-assembling peptide hydrogel.

64. The method of claim 62, wherein the peptides comprise amphiphilic peptides, and wherein the peptides comprise substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure.

65. The method of claim 59, wherein the nanoscale environment scaffold comprises nanofibers.

66. The method of claim 65, wherein the nanofibers are comprised of self-assembling peptides.

67. The method of claim 59, wherein the cells comprise progenitor cells.

68. The method of claim 59, wherein the cells comprise stem cells, which may be either adult stem cells or embryonic stem cells.

69. The method of claim 59, wherein the cells comprise liver-derived cells or cells derived from neural tissue.

70. The method of claim 59, wherein the cells comprise stem cells or progenitor cells that have been instructed or induced to differentiate.

71. The method of claim 70, wherein the cells are instructed or induced to differentiate along a liver cell lineage pathway.

72. The method of claim 71, wherein the cells comprises liver cells which may include liver stem cells, liver progenitor cells, hepatocytes, oval cell, bile duct cells, or a combination of the foregoing cell types.

73. The method of claim 70, wherein the cells are instructed or induced to differentiate along a neural cell lineage pathway.

74. The method of claim 73, wherein the cells comprise neurons, glia, or a combination of neurons and glia.

75. A nanoscale environment scaffold encapsulating cells.

76. The nanoscale environment scaffold encapsulating cells of claim 75, wherein the scaffold comprises a protein or peptide hydrogel.

77. The nanoscale environment scaffold encapsulating cells of claim 76 wherein the hydrogel is comprised of self-assembling peptides.

78. The nanoscale environment scaffold encapsulating cells of claim 77, wherein the peptides comprise amphiphilic peptides comprising substantially equal proportions of hydrophilic and hydrophobic amino acids, are complementary and structurally compatible, and are capable of self-assembling into a beta-sheet macroscopic structure.

79. The nanoscale environment scaffold encapsulating cells of claim 75, wherein the scaffold comprises nanofibers.

80. The nanoscale environment scaffold encapsulating cells of claim 79, wherein the nanofibers comprise self-assembling peptides.

81. The nanoscale environment scaffold encapsulating cells of claim 80, wherein the cells comprise progenitor cells or stem cells.

82. The nanoscale environment scaffold encapsulating cells of claim 75, wherein the cells comprise liver-derived cells or cells derived from neural tissue.

83. The nanoscale environment scaffold encapsulating cells of claim 75, wherein the cells comprise progenitor cells or stem cells that have been instructed or induced to differentiate.

84. The nanoscale environment scaffold encapsulating cells of claim 83, wherein the cells are instructed or induced to differentiate along a liver cell lineage pathway.

85. The nanoscale environment scaffold encapsulating cells of claim 84, wherein the comprise liver cells and may include liver stem cells, liver progenitor cells, hepatocytes, oval cell, bile duct cells, or combinations of the foregoing cell types.

86. The nanoscale environment scaffold encapsulating cells of claim 83, wherein the cells are instructed or induced to differentiate along a neural lineage pathway.

87. The nanoscale environment scaffold encapsulating cells of claim 86, wherein the cells comprise neural lineage cells which may include neurons, glia, or a combination of the foregoing cell types.

88. The nanoscale environment scaffold encapsulating cells of claim 75, wherein the cells comprise liver lineage cells and neural lineage cells.

89. A method of treating an individual comprising: (i) identifying an individual in need of treatment; and (ii) administering a nanoscale environment scaffold encapsulating cells to the individual.

90. A method of treating an individual comprising (i) identifying an individual in need of treatment; and (ii) administering the nanoscale environment scaffold encapsulating cells of any of claims 75, 78, or 81 to the individual.

91. A composition comprising insulin secreting cells encapsulated within a peptide hydrogel.

92. The composition of claim 91, wherein the insulin secreting cells secrete insulin at a level of at least ten percent of the level of normal pancreatic beta cell insulin secretion per milligram of total protein.

93. The composition of claim 91, wherein the insulin secreting cells secrete insulin at a level of at least twenty percent of the level of normal pancreatic beta cell insulin secretion per milligram of total protein.

94. A method of obtaining insulin secreting cells comprising steps of: culturing liver progenitor cells in a peptide hydrogel containing an extracellular matrix component selected from the group consisting of laminin and fibronectin in the presence of at least one growth factor selected from the group consisting of epidermal growth factor and basic fibroblast growth factor.

95. A method of treating an individual suffering from a condition characterized by an absolute or relative lack of insulin functional activity or characterized by insulin resistance, the method comprising steps of: administering to the individual a

composition comprising a peptide hydrogel and cells that have been induced to differentiate into insulin secreting cells within the peptide hydrogel.

96. A method of treating an individual suffering from a condition characterized by an absolute or relative lack of insulin functional activity or characterized by insulin resistance, the method comprising steps of: administering to the individual a composition comprising insulin secreting cells isolated from a peptide hydrogel.

97. The method of claim 95 or 96, wherein the condition is diabetes.

98. The method of claim 95 or 96, wherein the insulin secreting cells are derived from liver progenitor cells.

99. The macroscopic structure of claim 12, wherein the extracellular matrix component is laminin or fibronectin.

100. The macroscopic scaffold of claim 3, wherein at least a portion of the progenitor cells or their progeny differentiate or transdifferentiate into cells expressing a pancreatic cell marker.

101. The macroscopic scaffold of claim 100, wherein the pancreatic marker is insulin.

102. The macroscopic scaffold of claim 101, wherein the cells secrete insulin in response to a glucose stimulus.

103. The method of claim 34, wherein the extracellular matrix component is fibronectin or laminin.

104. The method of claim 26, wherein at least a portion of the progenitor cells or their progeny differentiate or transdifferentiate into cells expressing a pancreatic cell marker.

105. The method of claim 104, wherein the pancreatic cell marker is insulin.

106. The method of claim 70, wherein the cells are instructed or induced to differentiate along a pancreatic cell lineage pathway.

107. The nanoscale environment scaffold encapsulating cells of claim 83, wherein the cells are instructed or induced to differentiate along a pancreatic cell lineage pathway.

FIG. 1

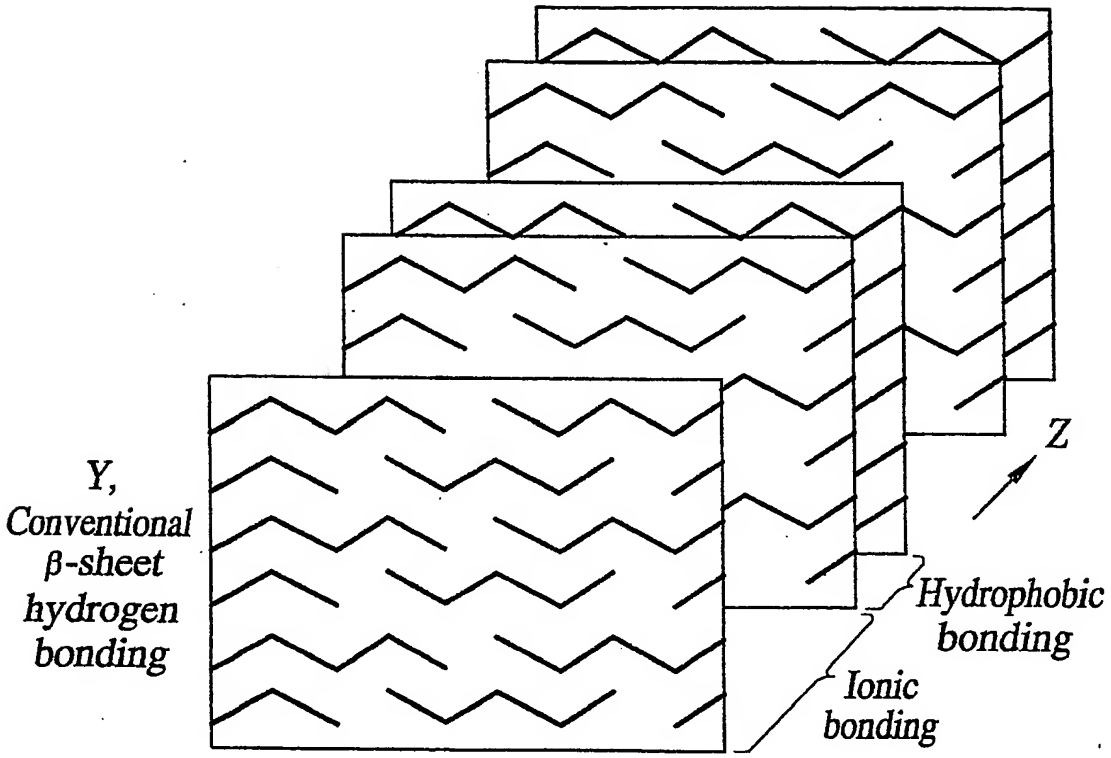

Y,
Conventional
β-sheet
hydrogen
bonding
Z
Hydrophobic
bonding
Ionic
bonding
X, Along the length of staggered peptides

FIG.2a

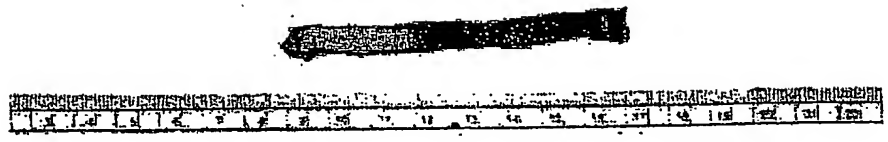


FIG.2b

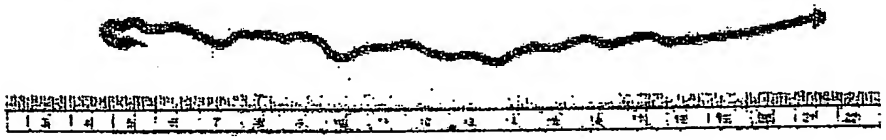
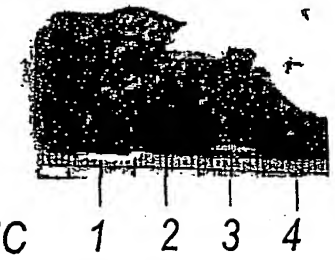


FIG.2c



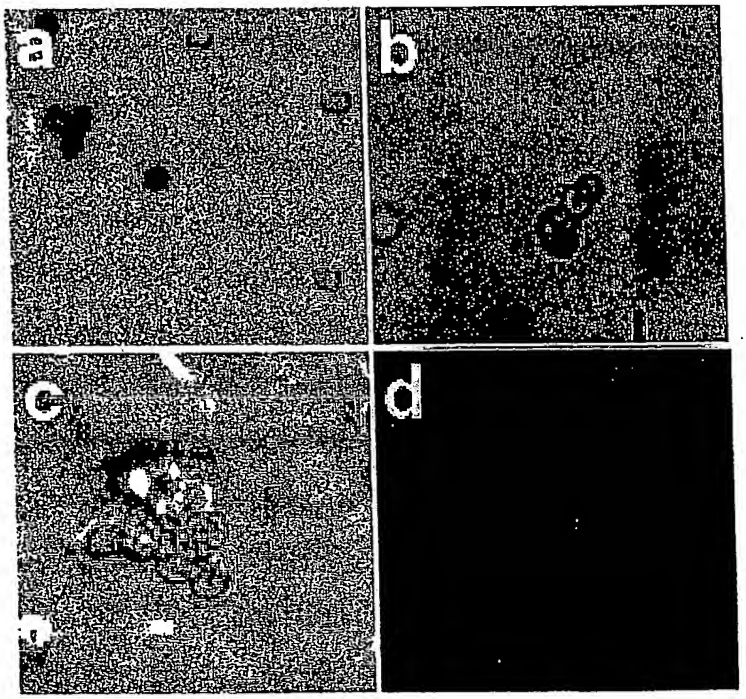


Figure 3

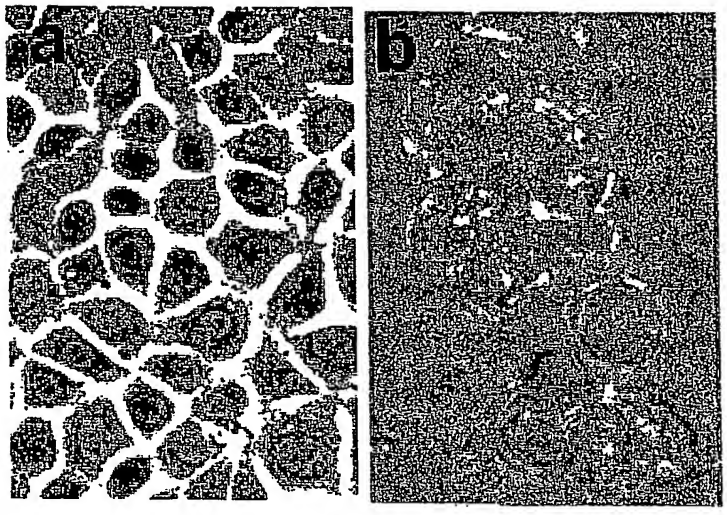


Figure 4

FIG.4c

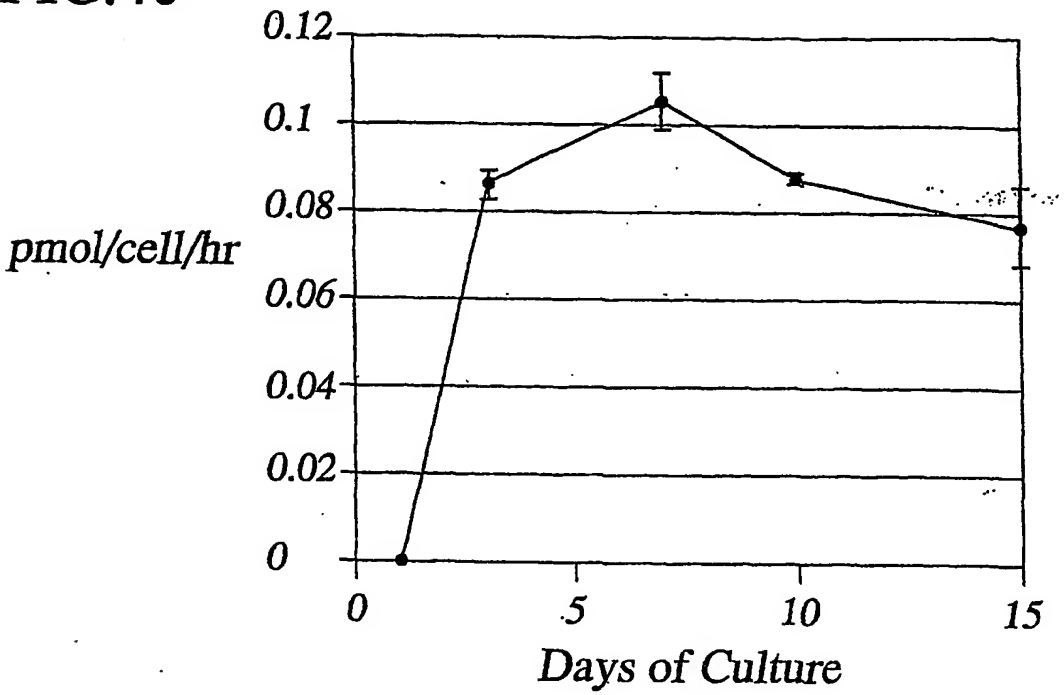
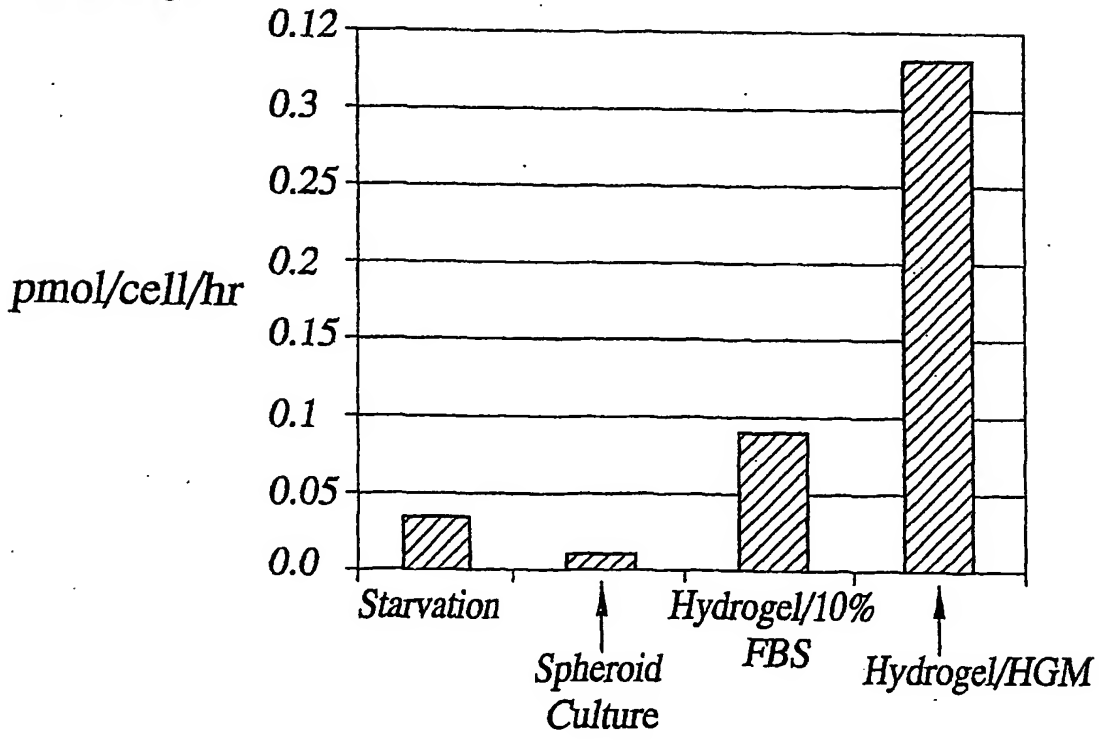

0.12
0.1
0.08
0.06
0.04
0.02
0
pmol/cell/hr
0
5
10
15
Days of Culture

FIG.4d


0.12
0.3
0.25
0.2
0.15
0.1
0.05
0.0
pmol/cell/hr
Starvation
Spheroid Culture
Hydrogel/10% FBS
Hydrogel/HGM

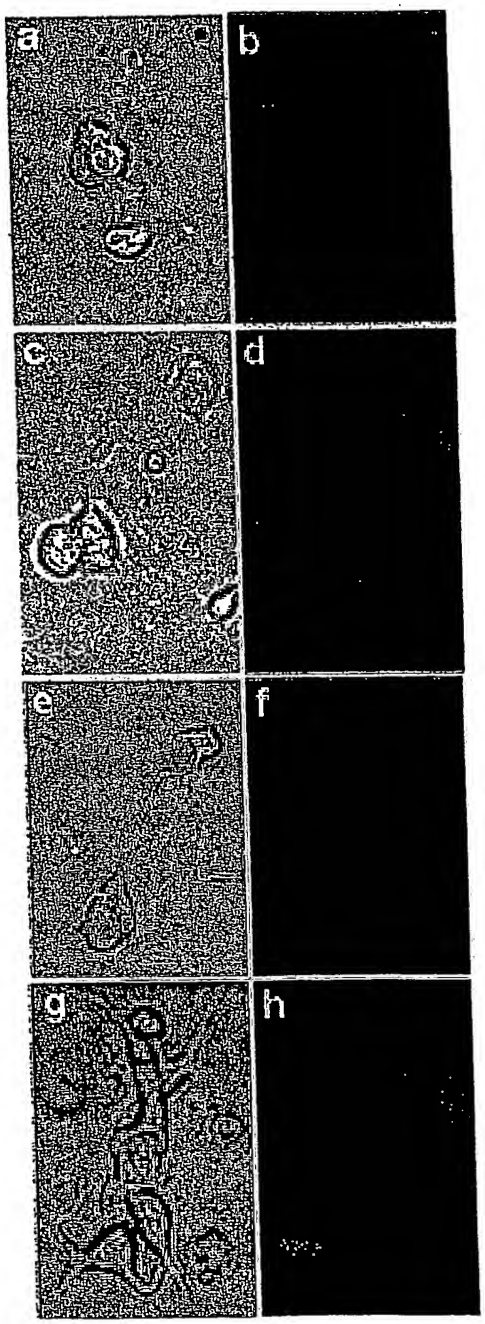


Figure 5

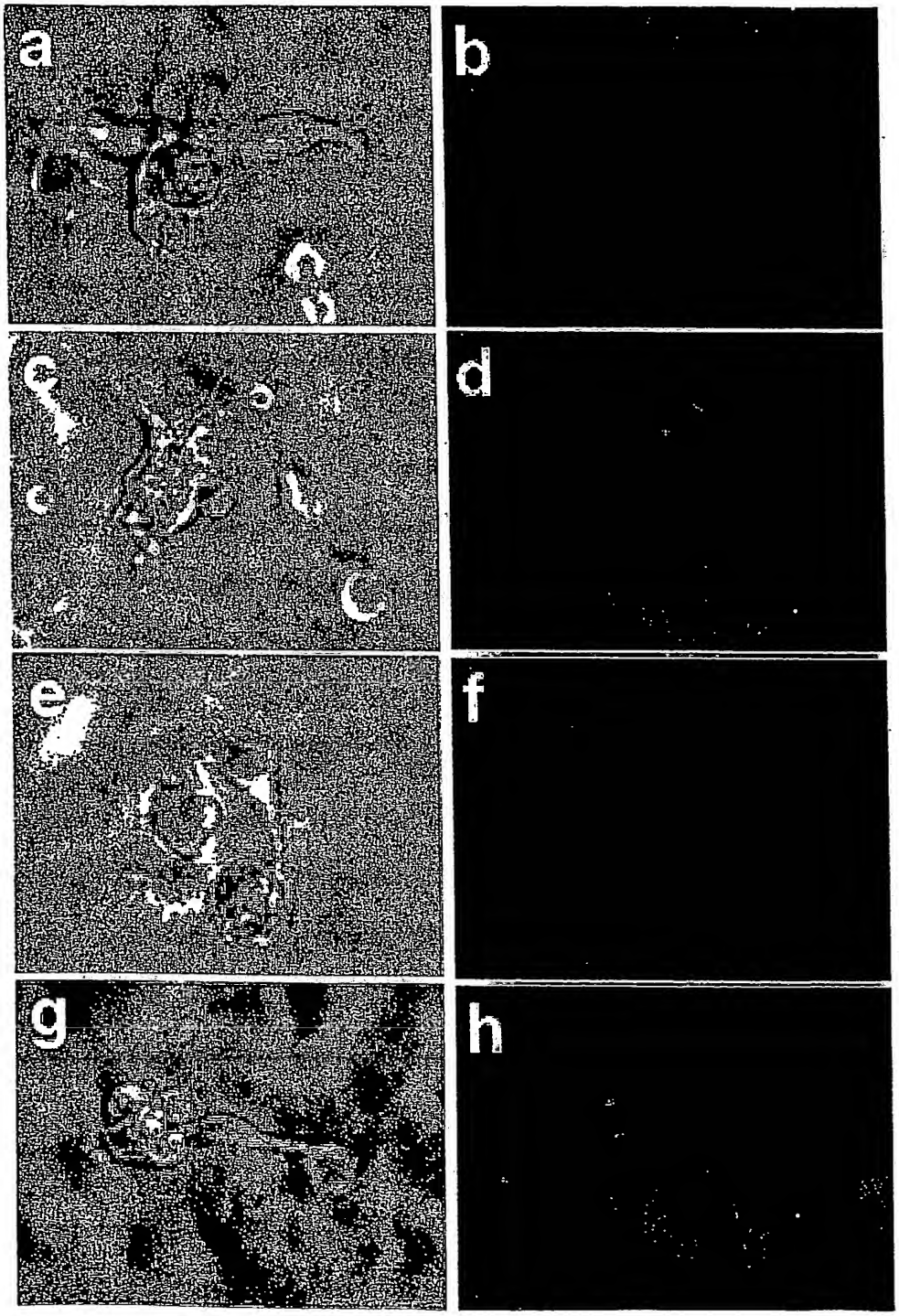


Figure 6

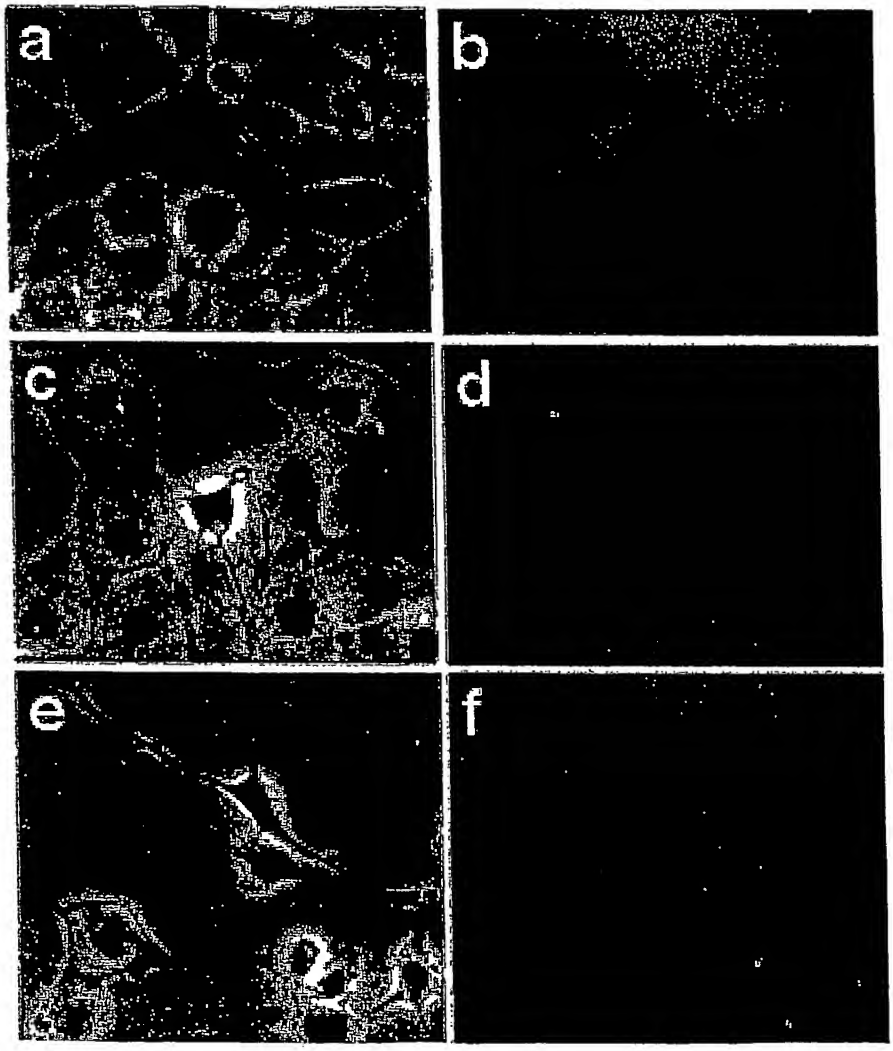


Figure 7

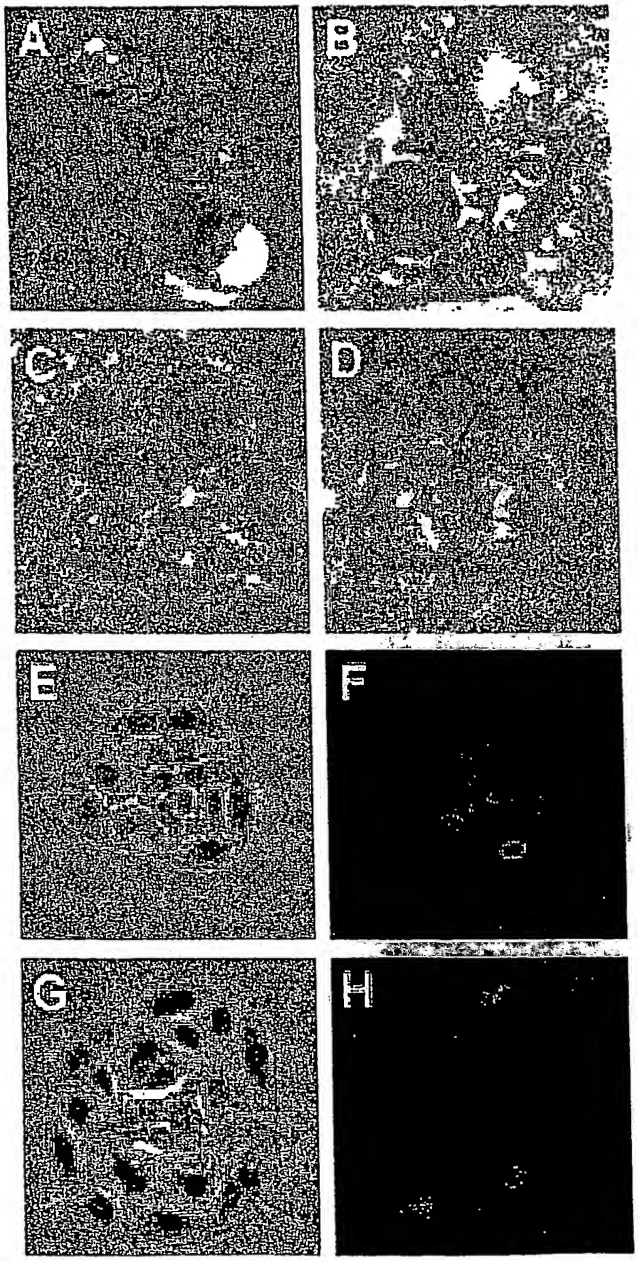


FIGURE 8

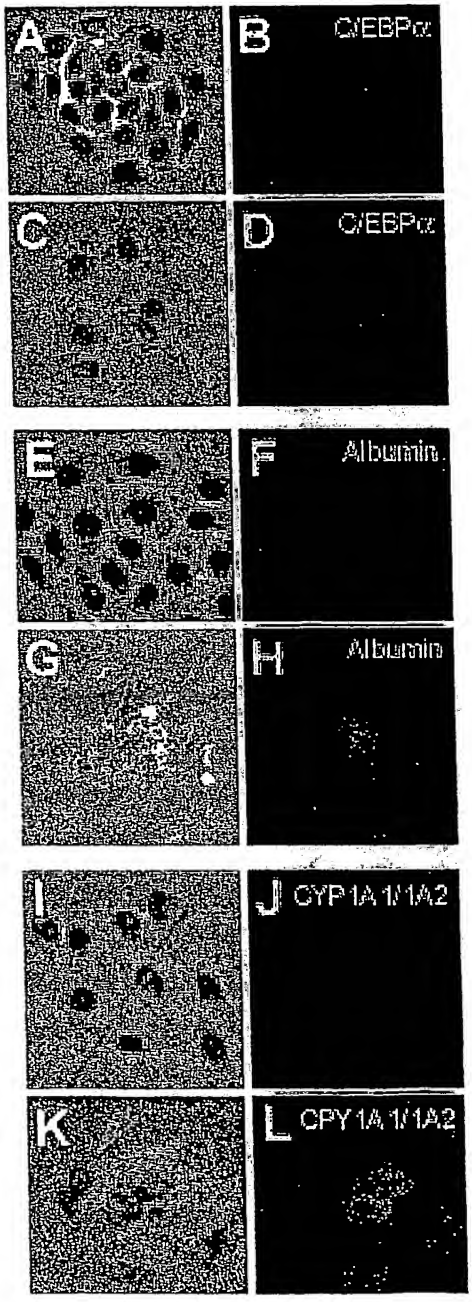

A
B
C/EBPα
C
D
C/EBPα
E
F
Albumin
G
H
Albumin
I
J
CYP1A1/1A2
K
L
CPY1A1/1A2

FIGURE 9

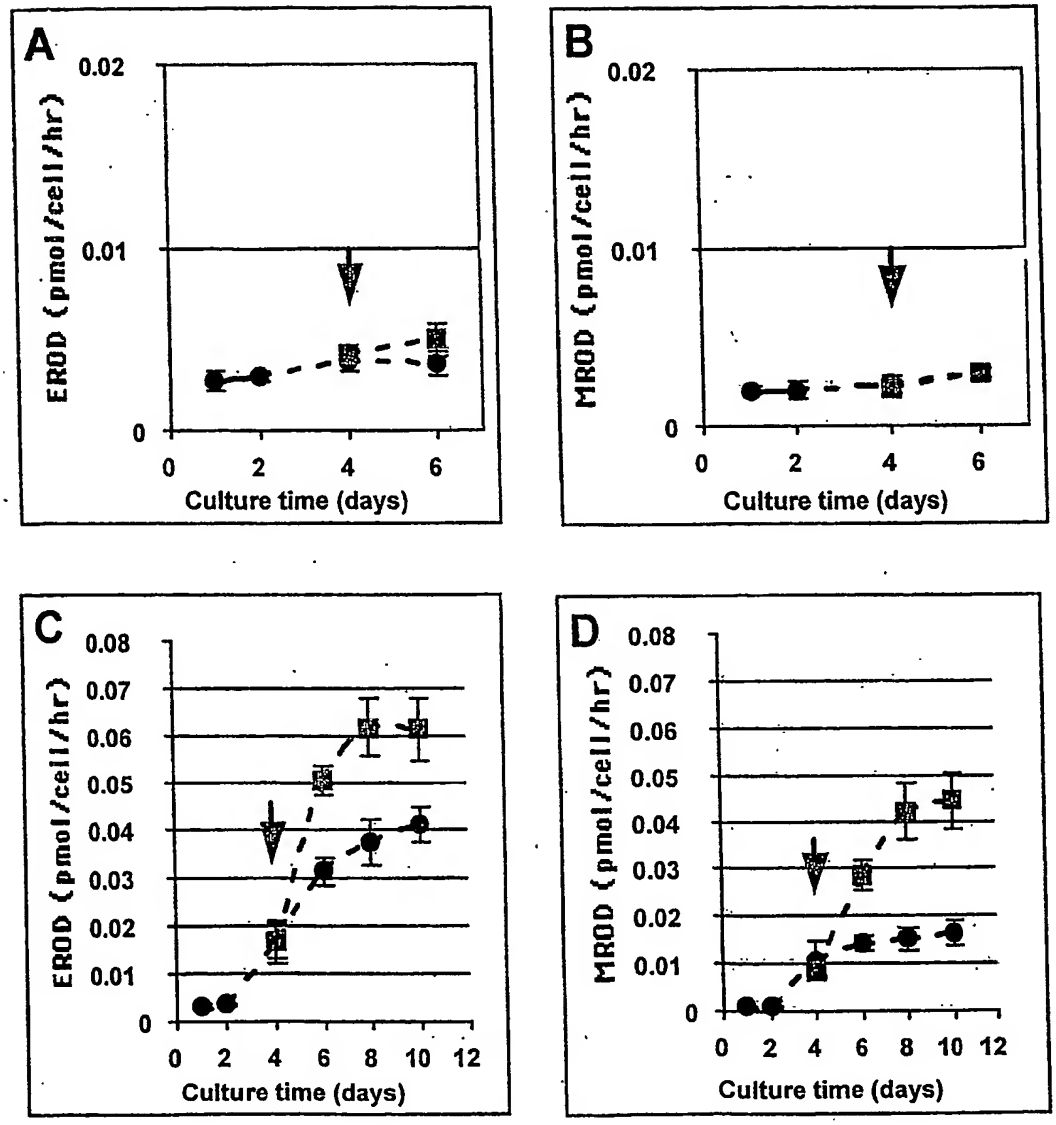

A
EROD (pmol/cell/hr)
0.02
0.01
0
0 2 4 6
Culture time (days)
B
MROD (pmol/cell/hr)
0.02
0.01
0
0 2 4 6
Culture time (days)
C
EROD (pmol/cell/hr)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0 2 4 6 8 10 12
Culture time (days)
D
MROD (pmol/cell/hr)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0 2 4 6 8 10 12
Culture time (days)

FIGURE 10

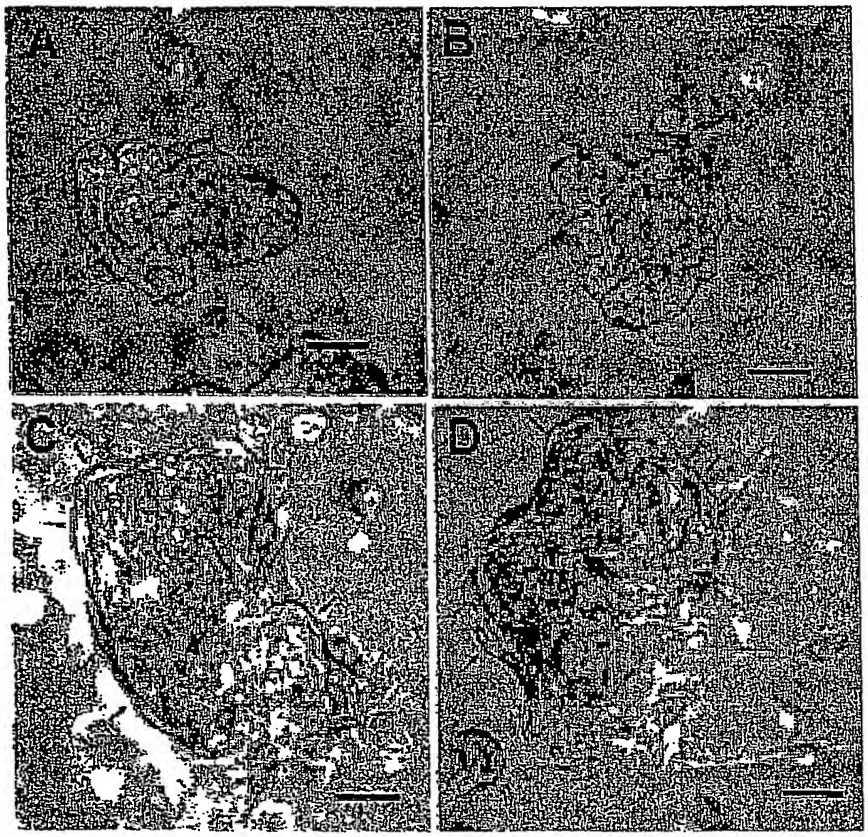


FIGURE 11

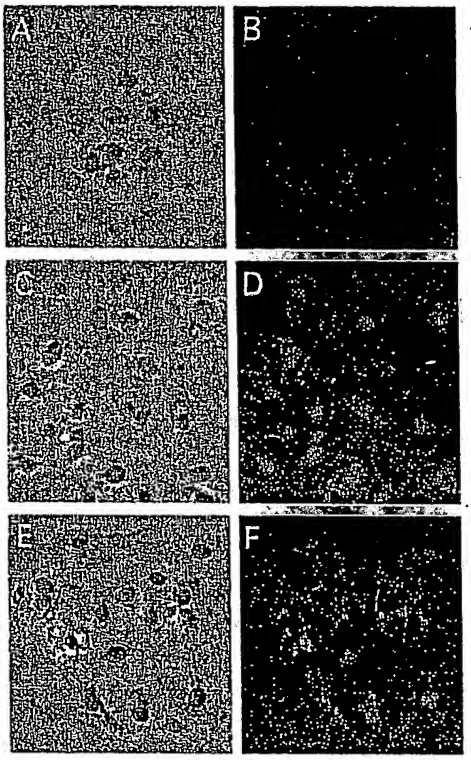
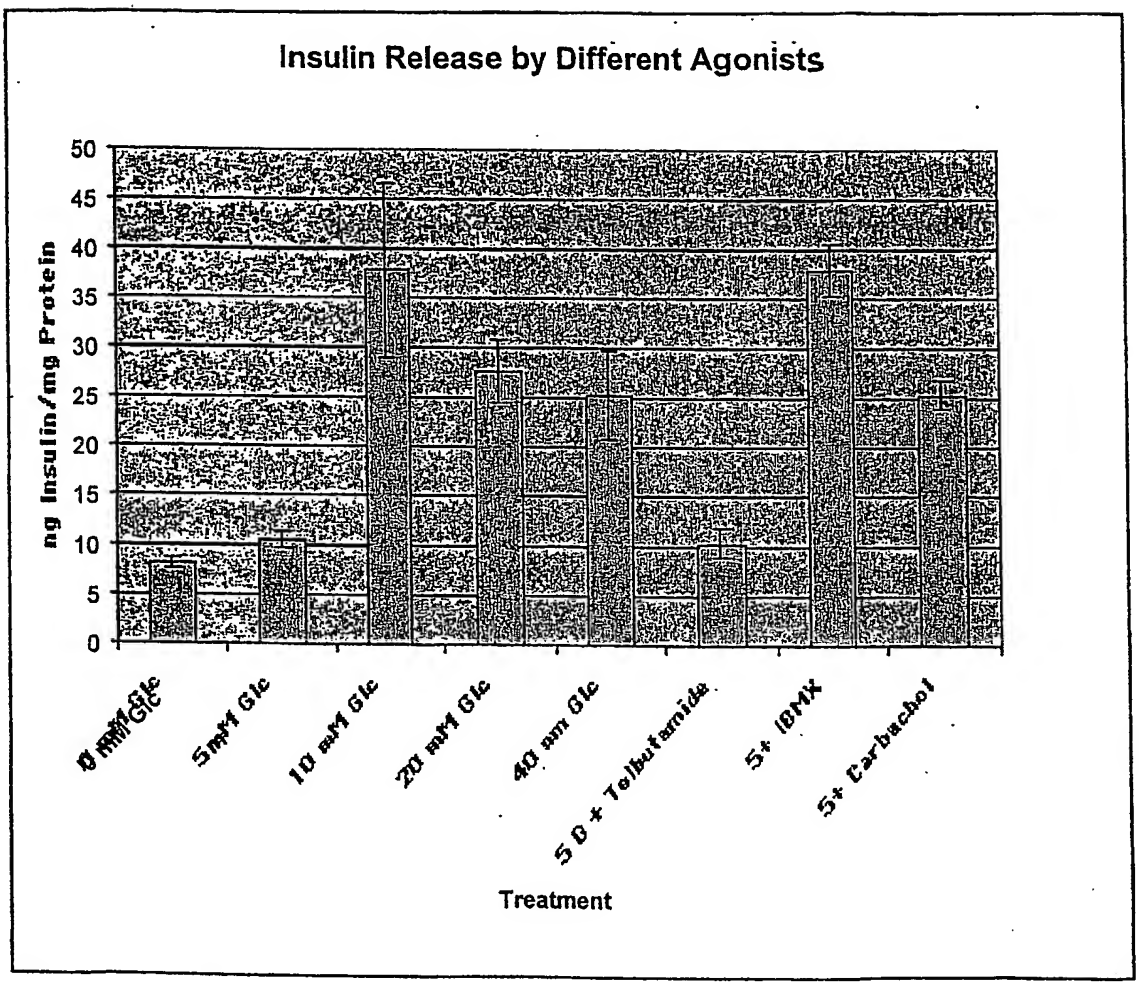


FIGURE 12



5+ = 5 mM glucose

FIGURE 13